Supplementary Information
For

Influence of stereochemistry on the activity of the rapadocin, an isoform-specific inhibitor of nucleoside transporter ENT1

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Table of Contents

BIOLOGY:

Biological Reagents

Swine kidney tubular epithelial cell line PK15, its derivatives PK15-ENT1 and PK15-ENT2 were provided by Dr. Chung-Ming Tse (Johns Hopkins University School of Medicine). Jurkat T cells were purchased from ATCC. Human red blood cells were obtained from Fisher Scientific (Cat#: 50-643-497). DMEM and RPMI-1640 medium were purchased from Fisher Scientific (Cat#: 11885092, Cat#: 11875119). [^{3}H]-thymidine was purchased from PekinElmer (Cat#: NET027). Vectors pET-28a(+) and pDEST15 were purchased from Novagen and Invitrogen, respectively. Ni-NTA agarose beads was purchased from Qiagen (Cat#: 30210). Hi-prep Q anion-exchange column, glutathione beads and sephacryl S-100 beads were purchased from GE life sciences (Cat#: 17115301, 17075601, and 17061210). Streptavidin agarose beads and glutathione magnetic beads were purchased from ThermoFisher Scientific (Cat#: 20359 and 78601). Protein assay kit was purchased from Bio-Rad (Cat#: 5000006). Antibody ENT1 was purchased from Santa Cruz Biotechnology (Cat#: SC377283).

Cell Culture

All cells were grown at 37°C with 5% CO_2 in a humidified environment. PK15-ENT1 and PK15-ENT2 cells were grown in DMEM media with the addition of 10% FBS. Jurkat T cells were grown in RPMI-1640 media with the addition of 10% FBS.

Proliferation Assay

15,000 Jurkat T wt or FKBP12 knockout cell/well was seeded in a 96-well plate (Costar) in 180 µL media; PK15-ENT1 or PK15-ENT2 cells were seeded at 10,000 cells/well. After an overnight

recovery, drugs were added. Following a 30min incubation, cells were pulsed with 1 µCi of [^{3}H]-thymidine for 30min, washed once with PBS, trypsinized, and transferred to filtermats using a Mach III M Harvester 96 (Tomtec). After drying, [^{3}H]-thymidine retention on the filtermats was determined by scintillation counting using a 1450 Microbeta apparatus (Wallac). Counts were normalized to vehicle only treated cells. GraphPad Prism (v4.03) software was used to determine IC_{50} values using a four-parameter logistic regression.

Overexpression and Purification of FKBP12 Protein

The FKBP1A coding cDNA was inserted into either the pET-28a (+) vector between NdeI and XhoI for his-tag fusion protein or pDEST15 using Gateway cloning technology (Invitrogen) for GST fusion protein. The cloned genes were confirmed not to contain any spurious mutations by sequencing the full length of the cloned inserts. The gene products were then expressed under induction of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4h at 37°C. The 6xHis-FKBP12 protein was purified from the crude extract prepared from the harvested cells, first by metal chelating chromatography using Ni-NTA agarose beads and then by a 5ml Hi-Prep Q anion-exchange column. The GST-FKBP12 protein was purified first by glutathione beads and then by size exclusion chromatography using sephacryl S-100 beads. Purified proteins were quantified by protein assay kit and stored at -20°C until used in PBS buffer (pH 7.4) plus 5 mM DTT and 10% glycerol.

Measurement of K_d Values for Rapafucins

Dissociation constant K_d values for rapafucins were determined with the fluorescence chemical denaturation assay as described previously [1]. Briefly, 1µM of purified FKBP12 protein bearing his-tag was incubated individually with 10µM of each rapafucins in 96-well plates and then equilibrated over a range of chemical denaturant concentrations (GuHCl, 0-4M). After an overnight incubation at RT, the fluorescence (ex 280nm / em 340nm) of FKBP12 or FKBP12-rapafucin-denaturant complexes was measured using a fluorescence plate reader, FLUOstar Optima (BMG Labtech, U.K.). Apparent K_d values for rapafucins were obtained using the data analysis method previously described[1].

Extraction of ENT1 Protein from Red Blood Cells

Human red blood cells were washed once in buffer A (10 mM Tris-HCl, 150 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.4), and membranes were prepared by dispersing 1 volume of cells in 24 ml ice cold buffer B (10 mM Tris-HCl, 1 mM EGTA, pH 7.4). After 10 min of incubation on ice, membranes were collected at 17,000 g for 10 min at 4°C. To extract Ent1 protein, membranes were mixed in buffer C (10 mM Tris-HCl, 150 mM KCl, 5 mM $MgCl_2$, 1% OTG, 5% glycerol, 1 mM EGTA, protease inhibitor, pH 7.4) and incubated on ice for 2 h with frequent mixing. The supernatant was collected by centrifugation at 17,000 g for 10min at 4°C, and then diluted four-fold in buffer A plus 5% glycerol to reduce the concentration of OTG. The diluted supernatant was quantified by protein assay kit and placed on ice until use.

GST-FKBP12 Affinity Pull-down

The supernatant containing extracted ENT1 protein was obtained from previous extraction step and pre-incubated with glutathione magnetic beads at 4°C for 30min to remove the endogenous

glutathione-binding proteins. The supernatant was collected by precipitation of beads on the magnet and diluted to 10mg/ml for pull-down experiment. For a typical GST-FKBP12 pull-down reaction, 300 µL of supernatant was pretreated with 20 µM of drugs or equal volume of DMSO (as noted in the text) for 30min, before the addition of 2µM of purified GST-FKBP12. After incubation at 4^{o}C for 1h with frequent mixing, 30 µl of glutathione-magnetic beads in buffer A plus 5% glycerol was added, and incubation was continued for 2h. The magnetic beads were precipitated on the magnet and washed three times with 0.8 ml of buffer A plus 5% glycerol and 0.05% OTG. The washed glutathione-magnetic beads was then resuspended in 50 µl of 2 x SDS sample buffer, heated at 100^{o}C for 5 min and centrifuged for 2 min. The supernatant was subjected to SDS-PAGE followed by western blot.

Western Blot Analysis

Cell lysates or samples from pull-down experiment were subjected to SDS/ PAGE and then transferred to a nitrocellulose membrane. Membranes were first blocked in 5% (wt/vol) BSA in Tris-buffered saline plus 0.1% Tween 20 (TBST) at room temperature for 30min and incubated with primary antibodies at 4 °C for overnight. Membranes were then washed three times with TBST and incubated with secondary antibodies at room temperature for another 1 h. Membranes were washed with TBST three times again and incubated with ECL substrate for 1 min at room temperature. Pictures were captured using a GeneSys Image Station.

CRISPR-Cas9 Knockout Protocol

To create knockout line, cells were transfected with CRISPR all-in-one plasmid (GeneCopoeia) containing transcripts for Cas9, guideRNA, and mCherry fluorescent protein. This was performed by electroporation of 10 million Jurkat T wild type cells in 400µl of FBS/antibiotic free RPMI 1640 and 10µg cDNA (0.4cm gap cuvette) at 250V and 950µF using a Bio-Rad Gene Pulser II. After 48h rest, cells were centrifuged, aspirated, and re-suspended in sorting buffer (1x PBS, 25mM HEPES, 1% FBS, 2% Penn-Strep). Cells were then sorted (single-cell) into three pre-filled 96-well plates (100uL of RPMI 1640 containing 10% FBS and 2% Penn-Strep) for mCherry fluorescence at 561nm. Single cell colonies were allowed to grow 1 week before addition of 100µL of RPMI 1640 containing 10%FBS and 1% Penn-Strep. After 2-3 weeks, visible colonies were transferred to T-25 flasks and grown to 10^5cells/mL before western blot for expression.

CHEMISTRY:

General Information

All chemical reagents were purchased from commercial suppliers without further purification. Normal phase disposable flash columns RediSep®Rf for flash chromatography with Teledyne Isco CombiFlash Rf 200 were purchased from Teledyne Isco, Inc. Solvent was used extra dry over molecular sieve, stabilized, AcroSeal®. Compound 11 and Z-FKBD part were provided by Affinity Research Chemicals, Inc at Wilmington, DE USA. Yields refer to chromatographically homogeneous materials. Reactions were monitored by Thin Layer Chromatography TLC Silica gel 60 F_{254} supplied by EMD Millipore visualized by UV or basic solution of $KMnO_4$. Mass

spectrometry provided by Agilent 6120 Quadrupole LC/MS and the column for HPLC co-injections was Agilent SB-C18, 1.8um, 2.1x50 mm, 827700-902, USWEY14619. NMR spectra were recorded on Bruker Avance III 500 MHz NMR spectrometer and calibrated by using TMS as internal standard for ^{1}H-NMR (0 ppm) and $CDCl_3$ for ^{13}C-NMR (77.0 ppm). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, b = broad, td = triple doublet, dt = double triplet, dq = double quartet, m = multiplet. High-resolution mass spectra (HRMS) were recorded on Waters Synapt G2-Si mass spectrometer using ESI (electrospray ionization).

LC-MS Analytical Protocol for Macrocycles

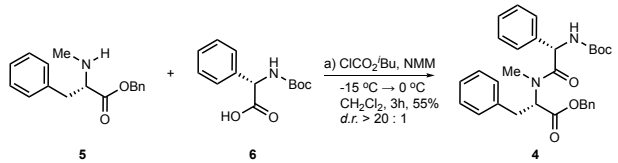
Analytical reversed-phase high-performance liquid chromatography (HPLC) was performed on a C-18 reverse phase HPLC column (5µm Luna, 0.46 cm × 25 cm). Separations were achieved using a linear gradient of 40%-95% buffer B in A (A = 0.1% formic acid in H_2O; B = 0.1% formic acid in CH_3CN) at a flow rate of 1 mL/min.

Screening for the diastereoselective synthesis of dipeptide 4

To a mixture of Boc-Phg-OH (0.100 mmol, 25 mg, 1.0 equiv), **5** (0.100 mmol, 27 mg, 1.0 equiv), in flame-dried 10 mL-Schlenk tube, dry DMF (1.0 mL) was added under Ar balloon protection, and base (1.0 equiv) and coupling reagents were added. The mixture was stirred at specific temperature for 3h. The ratio was detected by crude ^{1}H-NMR.

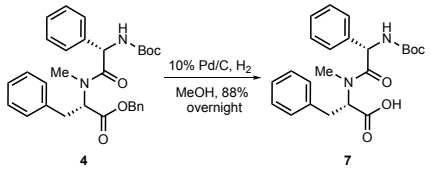
Experimental Procedures and Spectroscopic Data of the Synthesized Compounds

Synthesis of compound **4**:



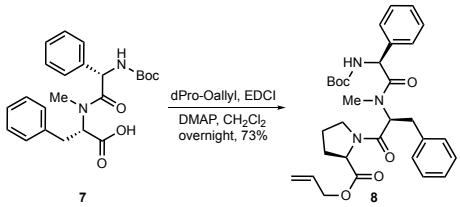
To a stirred solution of Boc-Phg-OH **6** (1.00 g, 4.0 mmol) and **5** (1.08 g, 4.0 mmol) in CH_2Cl_2 (12 mL) was added N-Methylmorpholine (NMM, 0.52 mL, 4.8 mmol) followed by Isobutyl choroformate (0.52 mL, 4.8 mmol) dropwise at -15 °C. After being stirred for 2 h, the resulting solution was quenched with a saturated solution of NH_4Cl (20mL). the reaction mixture was extracted three times with ethyl acetate (20 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by CombiFlash on disposable flash columns RediSep®Rf (pure hexane to hexane/ethyl acetate = 9/1) to yield **4** (1.10 g, 2.2 mmol) as white amorphous solid; $[\alpha]_D^{23}$= +3.8 (c = 1.0 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 7.36-7.26 (m, 8H), 7.22-7.13 (m, 5H), 7.05 (d, *J* = 7.1 Hz, 2H), 6.50 and 5.90 (rotamers, d, *J* = 7.8 Hz, 1H), 5.42 (d, *J* = 7.8 Hz, 1H), 5.19 (dd, *J* = 9.9, 5.5 Hz, 1H), 5.13 (d, *J* = 12.3 Hz, 1H), 5.05 (d, *J* = 12.2 Hz, 1H), 3.36(dd, *J* = 14.5, 5.5 Hz, 1H), 3.00 (dd, *J* =14.4, 10.1 Hz, 1H), 2.66 (s, 3H), 1.39(s, 9H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 170.5, 170.1, 154.9, 137.2, 136.7, 135.4, 128.9, 128.8, 128.6, 128.3, 128.3,128.2, 128.2, 127.9, 126.7, 79.7, 67.0, 59.8, 55.2, 34.5, 33.2, 28.4 ppm; HRMS (ESI): m/z calcd for $C_{30}H_{35}N_2O_5[M + H]^+$: 503.2546, found 503.2545.

Synthesis of compound **7**:



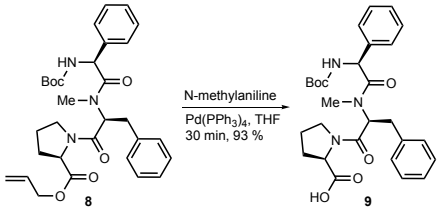
To a solution of compound **4** (357 mg, 0.71 mmol) in MeOH (5.0 mL) was added 10% Pd/C (50 mg, 0.02 equiv), and the resultant mixture was first degassed with hydrogen, and then stirred at room temperature for overnight. The reaction was quenched by filtration of the mixture through filter paper and washed with EtOAc (3 × 10 mL). The filtrate was concentrated under vacuum, and the residue was purified by CombiFlash on disposable flash columns RediSep®Rf (pure hexane to hexane/ethyl acetate = 4/1) to yield **7** (259 mg, 0.63 mmol) as white amorphous solid; $[\alpha]_D^{23}$= +5.3 (c = 0.68 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 10.6 (br s, 1H), 7.37-7.04 (m, 10H), 6.57 and 6.14 (rotamers, s, 1H), 5.53 (s, 1H), 5.14 (s, 1H), 3.37 (d, *J* = 13.9 Hz, 1H), 3.03-2.96 (m, 1H), 2.71(s, 3H), 1.38 (s, 9H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 173.6, 171.0, 155.0, 136.7, 136.6, 128.7, 128.6, 128.4, 128.1, 127.7, 126.5, 79.8, 59.8, 55.0, 34.0, 33.3, 28.2 ppm; HRMS (ESI): m/z calcd for $C_{23}H_{28}N_2O_5Na[M + Na]^+$: 435.1896, found 435.1898.

Synthesis of compound **8**:



To a stirred solution of compound **7** (93 mg, 0.22 mmol) and dPro-Oallyl (42 mg, 0.27 mmol) in CH_2Cl_2 (1.0 mL) was added DMAP (27 mg, 0.22 mmol) followed by EDCI (69 mg, 0.36 mmol) in a portion at room temperature and stirred for overnight. The reaction mixture was directly poured on disposable flash columns RediSep®Rf for further purification (pure hexane to hexane/ethyl acetate = 4/1) to yield **8** (88 mg, 0.16 mmol) as white amorphous solid; $[\alpha]_D^{23}$= +4.5 (c = 1.04 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 7.39-7.18 (m, 10H), 5.95-5.79 (m, 2H), 5.54-5.17 (m, 4H), 4.50 (s, 2H), 4.31 (s, 1H), 3.33 (s, 1H), 3.08 (s, 1H), 3.00 (s, 3H), 2.87-2.77 (m, 2H), 2.00(s, 1H), 1.76-1.52 (m, 3H) 1.41 (s, 9H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 171.1, 170.2, 167.4, 154.6, 137.1, 137.1, 131.5, 129.4, 128.6, 128.1, 128.0, 127.4, 126.3, 118.2, 79.4, 65.3, 58.6, 56.8, 54.8, 45.8, 34.8, 30.6, 28.5, 28.1, 24.4 ppm; HRMS (ESI): m/z calcd for $C_{31}H_{40}N_3O_6[M + H]^+$: 550.2917, found 550.2919.

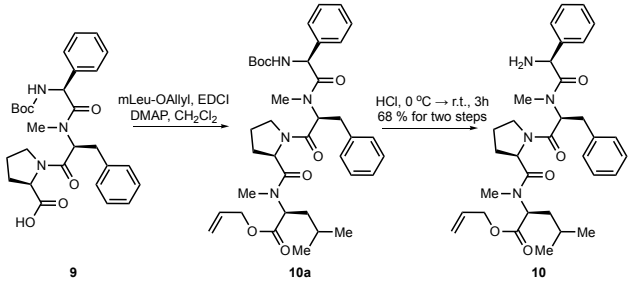
Synthesis of compound **9**:



To a stirred solution of compound **8** (144 mg, 0.26 mmol) and N-methylaniline (0.28 mL, 2.6 mmol) in THF (3.0 mL) was added $(PPh_3)_4Pd$ (30 mg, 0.026 mmol) in a portion, and the resultant

mixture was first degassed with Ar, and then stirred at room temperature for 30 minutes. The reaction was quenched with a solution of 1N HCl (5.0 mL). the reaction mixture was extracted two times with ethyl acetate (10 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by CombiFlash on disposable flash columns RediSep®Rf (pure hexane to hexane/ethyl acetate = 4/1 to 3/2) to yield **9** (124 mg, 0.24 mmol)) as white amorphous solid; $[\alpha]_D^{23}$= +54.9 (c = 0.86 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 9.75 (br s, 1H), 7.41-7.04 (m, 10H), 6.06 (s, 1H), 5.78 (s, 1H), 5.34 (s, 1H), 4.35 (s, 1H), 3.35 (t, *J* = 10.4 Hz, 1H), 3.08 (s, 3H), 2.98-2.80 (m, 2H), 2.66(s, 1H), 2.00-1.78 (m, 2H), 1.64-1.48 (m, 2H), 1.42 (s, 9H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 174.0, 170.7, 168.4, 155.0, 137.1, 137.1, 129.5, 128.7, 128.2, 128.2, 127.4, 126.5, 80.2, 58.7, 57.4, 54.4, 46.0, 34.9, 31.2, 28.3, 28.2, 24.5 ppm; HRMS (ESI): m/z calcd for $C_{28}H_{36}N_3O_6[M + H]^+$: 510.2604, found 510.2600.

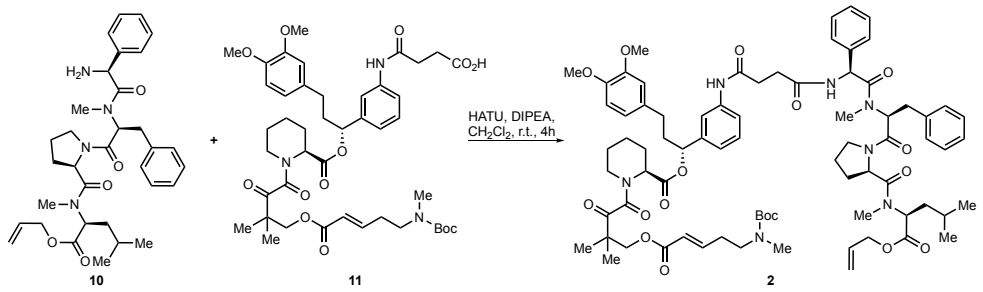
Synthesis of compound **10**:



To a stirred solution of compound **9** (76 mg, 0.15 mmol) and mLeu-Oallyl (40 mg, 0.18 mmol) in CH_2Cl_2 (1.0 mL) was added DMAP (18 mg, 0.15 mmol) followed by EDCI (46 mg, 0.24 mmol) in a portion at room temperature and stirred for 4h. The reaction mixture was directly poured on disposable flash columns RediSep®Rf for the further purification (pure hexane to hexane/ethyl acetate = 4/1 to 1/1) to yield **10a** (80 mg, 0.12 mmol) as white amorphous solid.

To a flame-dried flask was added **10a** (80 mg, 0.12 mmol), followed by the addition of HCl (1.0 mL, 4.0 M in dioxane) at 0 °C. After being stirred for 3 h, the reaction mixture was concentrated in vacuo. The residue was quenched with a saturated solution of $NaHCO_3$ (5.0 mL). the reaction mixture was extracted three times with ethyl acetate (5.0 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by CombiFlash on disposable flash columns RediSep®Rf (pure CH_2Cl_2 to CH_2Cl_2/(CH_2Cl_2/EtOH/MeOH = 2/2/1) = 9/1 to 7/3) to yield **10** (61 mg, 0.10 mmol)) as white amorphous solid; $[\alpha]_D^{23}$= -52.2 (c = 0.36 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 7.35-7.17 (m, 10H), 5.94-5.82 (m, 1H), 5.60-5.48 (m, 1H), 5.37-5.17 (m, 2H), 4.97 and 4.72 (rotamers, dd, *J* = 9.4, 6.4 Hz, 1H), 4.66-4.55 (m, 3H), 3.42-2.77 (m, 10H), 2.20-1.39 (m, 10H), 1.02-0.86 (m, 6H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 173.2, 172.0, 171.1, 167.3, 140.2, 137.7, 131.9, 129.6, 128.9, 128.8, 128.1, 127.0, 126.7, 118.0, 65.4, 56.8, 56.2, 55.4, 46.2, 37.4, 34.9, 32.1, 30.6, 28.2, 24.9, 24.6, 23.1, 21.2 ppm; HRMS (ESI): m/z calcd for $C_{33}H_{45}N_4O_5[M + H]^+$: 577.3390, found 577.3389.

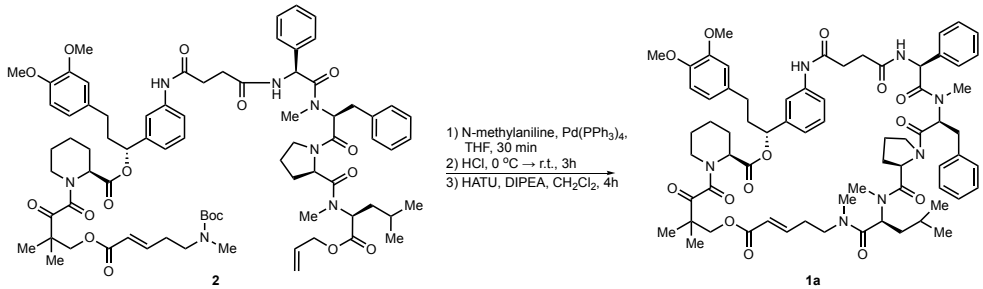
Synthesis of compound **2**:



10 11 2

To a stirred solution of compound **10** (27 mg, 0.047 mmol) and compound **11** (39 mg, 0.047 mmol) in CH_2Cl_2 (1.0 mL) was added DIPEA (24 µL, 0.14 mmol) followed by HATU (27 mg, 0.070 mmol) in a portion at room temperature and stirred for 4h. The resulting solution was quenched with a saturated solution of NH_4Cl (3.0 mL). the reaction mixture was extracted three times with ethyl acetate (3.0 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by CombiFlash on disposable flash columns RediSep®Rf (pure CH_2Cl_2 to CH_2Cl_2/(CH_2Cl_2/EtOH/MeOH = 2/2/1) = 17/3) to yield **2** (61 mg, 0.043 mmol) as white amorphous solid; $[\alpha]_D^{23}$= -5.5 (c = 0.53 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 8.64 and 8.58 (rotamers, s, 1H), 7.59-7.31 (m, 4H), 7.30-7.08 (m, 10H), 7.01 (d, *J* = 7.4 Hz, 1H), 6.96-6.86 (m, 1H), 6.80-6.74 (m, 1H), 6.71-6.65 (m, 2H), 5.93-5.83 (m, 1H), 5.81-5.75 (m, 2H), 5.48-5.34 (m, 1H), 5.33-5.07 (m, 3H), 4.97 and 4.69 (rotamers, m, 1H), 4.62-4.55 (m, 2H), 4.35-4.20 (m, 2H), 3.85 (s, 3H), 3.84 (s, 3H), 3.46 (d, *J* = 13.8 Hz, 1H), 3.39-3.23 (m, 3H), 3.18-3.05 (m, 3H), 2.99 (d, *J* = 5.8 Hz, 3H), 2.96-2.77 (m, 7H), 2.65-2.32 (m, 9H), 2.27-2.17 (m, 1H), 2.12-1.90 (m, 3H), 1.82-1.55 (m, 8H), 1.50-1.39 (m, 11H), 1.38-1.23 (m, 8H), 1.02-0.85 (m, 6H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 205.2, 171.9, 171.1, 170.9, 170.3, 169.8, 169.3, 168.1, 167.0, 166.2, 165.5, 148.7, 147.2, 140.5, 138.7, 137.4, 136.4, 133.4, 131.8, 129.7, 129.5, 129.1, 129.0, 128.9, 128.8, 128.2, 128.1, 127.7, 126.4, 122.1, 120.0, 118.1, 111.6, 111.2, 68.9, 65.9, 65.7, 65.5(3), 65.5(1), 57.8, 57.2, 57.1, 56.9, 55.8, 55.7, 55.5, 53.9, 51.2, 46.6, 46.5, 46.2, 46.1, 44.0, 38.4, 37.4, 35.0, 32.5, 32.1, 28.3, 28.2, 27.7, 26.2, 24.9, 24.5, 23.2, 21.7, 21.2, 20.9 ppm; HRMS (ESI): m/z calcd for $C_{77}H_{102}N_7O_{17}[M + H]^+$: 1396.7332, found 1396.7319.

Synthesis of rapadocin **1a**:



2 1a

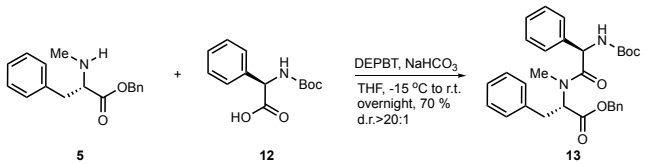
To a stirred solution of compound **2** (52 mg, 37.2 µmol) and N-methylaniline (40 µL, 372 µmol) in THF (0.50 mL) was added $(PPh_3)_4Pd$ (4.3 mg, 3.72 µmol) in a portion, and the resultant mixture was first degassed with Ar, and then stirred at room temperature for 30 minutes. The reaction was quenched with a solution of 1N HCl (3.0 mL). the reaction mixture was extracted two times with ethyl acetate (5 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by

CombiFlash on disposable flash columns RediSep®Rf (pure CH_2Cl_2 to CH_2Cl_2/ (CH_2Cl_2/EtOH/MeOH = 2/2/1) = 17/3) to get white amorphous solid (30.2 mg, 22.3 μmol);

To a flame-dried flask was added previous product (30.2 mg, 22.3 μmol), followed by the addition of HCl (1.0 mL, 4.0 M in dioxane) at 0 °C. After being stirred for 3 h, the reaction mixture was concentrated in vacuo and directly move to the next step.

To a stirred solution of previous product (22.3 μmol) in CH_2Cl_2 (0.5 mL) was added DIPEA (11 μL, 66.9 μmol) followed by HATU (12 mg, 44.6 μmol) in a portion at room temperature and stirred for 4h. The resulting solution was quenched with a saturated solution of NH_4Cl (3.0 mL). the reaction mixture was extracted three times with ethyl acetate (3.0 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by CombiFlash on disposable flash columns RediSep®Rf (pure CH_2Cl_2 to CH_2Cl_2/(CH_2Cl_2/EtOH/MeOH = 2/2/1) = 17/3) to yield **1a** (26.6 mg, 21.5 μmol) as white amorphous solid; $[\alpha]_D^{23}$= -36.5 (c = 0.94 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 8.97-8.91 and 7.93-7.73 (rotamers, m, 1H), 7.46-7.29 (m, 4H), 7.25-7.14 (m, 6H), 7.12-6.84 (m, 3H), 6.81-6.75 (m, 1H), 6.71-6.63 (m, 2H), 6.03-5.73 (m, 3H), 5.47-5.12 (m, 3H), 4.66-4.47 (m, 1H), 4.46-4.16(m, 2H), 3.86-3.85 (m, 6H), 3.75-3.56 (m, 1H), 3.46-3.35(m,1H), 3.30-3.10 (m, 3H), 3.06-2.82 (m, 9H), 2.80- 2.44 (m,9H), 2.40-2.17 (m, 3H), 2.12-1.91 (m, 2H), 1.89-1.38 (m, 14H), 1.37-1.18 (m, 7H),0.92-0.87 (m, 6H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 204.8, 171.1, 170.9, 170.6, 169.9, 169.7, 169.4, 167.5, 167.0, 166.0, 148.8, 147.3, 147.0, 145.8, 141.7, 138.9, 137.6, 136.6, 133.4, 129.6, 129.1, 128.9, 128.3, 128.2, 127.6, 127.5, 122.5, 122.3, 120.1, 117.0, 111.6, 111.2, 76.4, 69.3, 57.4, 55.9, 54.0, 53.6, 51.3, 50.2, 47.5, 46.9, 46.7, 46.3, 44.7, 44.1, 38.2, 37.7, 34.7, 34.1, 33.8, 31.2, 31.1, 30.3, 29.8, 29.6, 28.1, 25.0, 24.6, 23.0, 22.6, 21.7, 21.6, 21.0, 20.8 ppm; HRMS (ESI): m/z calcd for $C_{69}H_{88}N_7O_{14}$[M + H]$^+$: 1238.6389, found 1238.6364.

Synthesis of compound **13**:

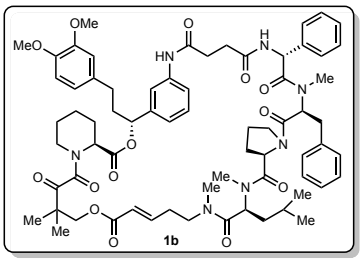


5 **12** **13**

To a stirred solution of Boc-dPhg-OH **12** (1.35 g, 5.0 mmol) and **5** (1.88 g, 7.5 mmol) in THF (30 mL) was added $NaHCO_3$ (420 mg, 5.0 mmol) followed by DEPBT (2.39 g, 8.0 mmol) in a portion at -15 °C. After being stirred for overnight and warmed to room temperature, the resulting solution was quenched with a saturated solution of NH_4Cl (40mL). the reaction mixture was extracted three times with ethyl acetate (50 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by CombiFlash on disposable flash columns RediSep®Rf (pure hexane to hexane/ethyl acetate = 9/1) to yield **13** (1.75 g, 3.5 mmol) as white amorphous solid; $[\alpha]_D^{23}$= -100.2 (c = 2.83 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 7.40-7.28 (m, 5H), 7.25-7.05 (m, 8H), 6.94 (d, *J* = 6.4 Hz, 2H), 5.88 (d, *J* = 6.4 Hz, 1H), 5.47 (d, *J* = 7.9 Hz, 1H), 5.36 (dd, *J* = 10.7 Hz, 4.0 Hz, 1H), 5.20 (s, 2H), 3.37 (d, *J* = 14.4 Hz, 1H), 3.00-2.90 (m, 1H), 2.63 (s, 3H), 1.40 (s, 9H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 170.9, 170.2, 155.0, 137.2, 136.5, 135.4, 128.9, 128.7, 128.4, 128.3, 127.8, 127.6, 126.8, 79.7, 67.2, 59.3, 55.6, 34.6, 32.9, 28.4 ppm; HRMS (ESI): m/z calcd for $C_{30}H_{35}N_2O_5$[M + H]$^+$: 503.2546, found 503.2555.

Synthesis of rapadocin **1b**:

Compound **1b** was synthesized according to the procedures for the synthesis of **1a**.



$[\alpha]_D^{23}$= -71.3 (c = 1.04 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.49-8.41 and 7.85-7.51 (rotamers, m, 1H), 7.30-7.01(m, 9H), 6.99-6.75 (m, 5H), 6.70-6.64 (m, 2H), 5.89-5.64 (m, 3H), 5.44-5.12 (m, 2H), 4.82-4.61 (m, 1H), 4.44-3.96 (m, 1H), 3.86-3.84 (m,6H), 3.78-3.64 (m, 1H), 3.60-3.27 (m, 3H), 3.23-2.58 (m, 16H), 2.54-2.00 (m, 11H), 1.93-1.53 (m, 7H), 1.51-1.12 (m, 13H), 0.92-0.84 (m, 6H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 204.0, 171.8, 171.4, 170.9, 170.3, 169.8, 169.6, 167.8, 166.5, 166.1, 148.8(4), 148.8(1), 147.3, 140.5, 138.9, 136.4, 133.4, 129.2, 129.1(6), 129.1(1), 129.0, 128.9, 128.4, 128.0, 127.5, 126.6, 122.7, 122.5, 121.8, 120.1, 111.6, 111.3, 76.2, 69.6, 69.3, 68.8, 60.4, 57.3, 55.9, 55.8, 53.9, 51.8, 51.5, 51.2, 47.4, 47.1, 46.6, 44.0, 38.2, 37.8, 34.9, 34.6, 33.7, 31.2, 30.2, 29.6, 28.4, 25.0, 24.7, 23.1, 22.2, 21.6, 21.3, 21.1, 14.1 ppm; HRMS (ESI): m/z calcd for $C_{69}H_{88}N_7O_{14}$[M + H]$^+$: 1238.6389, found 1238.6392.

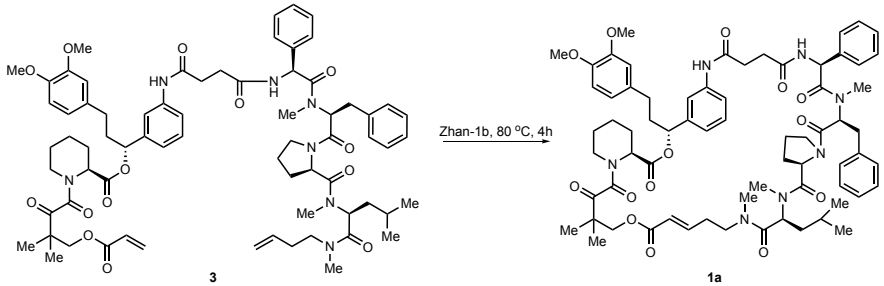
Synthesis of rapadocin **1c**:

Compound **1c** was synthesized according to the procedures for the synthesis of **1a**. However, the yield of cyclization step is low (about 5% after flash column, PTLC and HPLC purification), which means the Z configuration of the double bond is highly unfavorable for the conformation of rapafucins family molecules. Due to the low yield, we only collected ^{1}H NMR and HRMS.
HRMS (ESI): m/z calcd for $C_{69}H_{88}N_7O_{14}$[M + H]$^+$: 1238.6389, found 1238.6360.

Synthesis of rapadocin **1d**:

Compound **1d** was synthesized according to the procedures for the synthesis of **1b**. However, the yield of cyclization step is low (about 5%) after flash column, PTLC and HPLC purification, which means the Z configuration of the double bond is highly unfavorable for the conformation of rapafucins family molecules. Due to the low yield, we only collect ^{1}H NMR and HRMS.
HRMS (ESI): m/z calcd for $C_{69}H_{88}N_7O_{14}$[M + H]$^+$: 1238.6389, found 1238.6379.

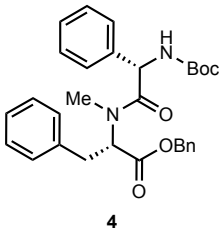
Synthesis of rapadocin **1a** from RCM strategy:



Compound **3** was synthesized according to the procedures for the synthesis of **2**. To a solution of compound **3** (25.0 mg, 20.0 µmol) in 1,2-dichloroethane (1.0 mL) was added **Zhan-1b** (2.5 mg, 4.0 µmol) catalyst, and the resultant mixture was stirred at 80 °C for 4h. The reaction mixture was directly poured on disposable flash columns RediSep®Rf for the further purification (pure CH_2Cl_2 to CH_2Cl_2/(CH_2Cl_2/EtOH/MeOH = 2/2/1) = 17/3) to yield **1a** (18.1 mg, 14.6 mmol) as white amorphous solid; $[\alpha]_D^{23}$= -35.9 (c = 0.59 in CH_2Cl_2); HRMS (ESI): m/z calcd for $C_{69}H_{88}N_7O_{14}$[M + H]$^+$: 1238.6389, found 1238.6377.

Spectra of the synthesized compounds

Compound 4: ^{1}H-NMR



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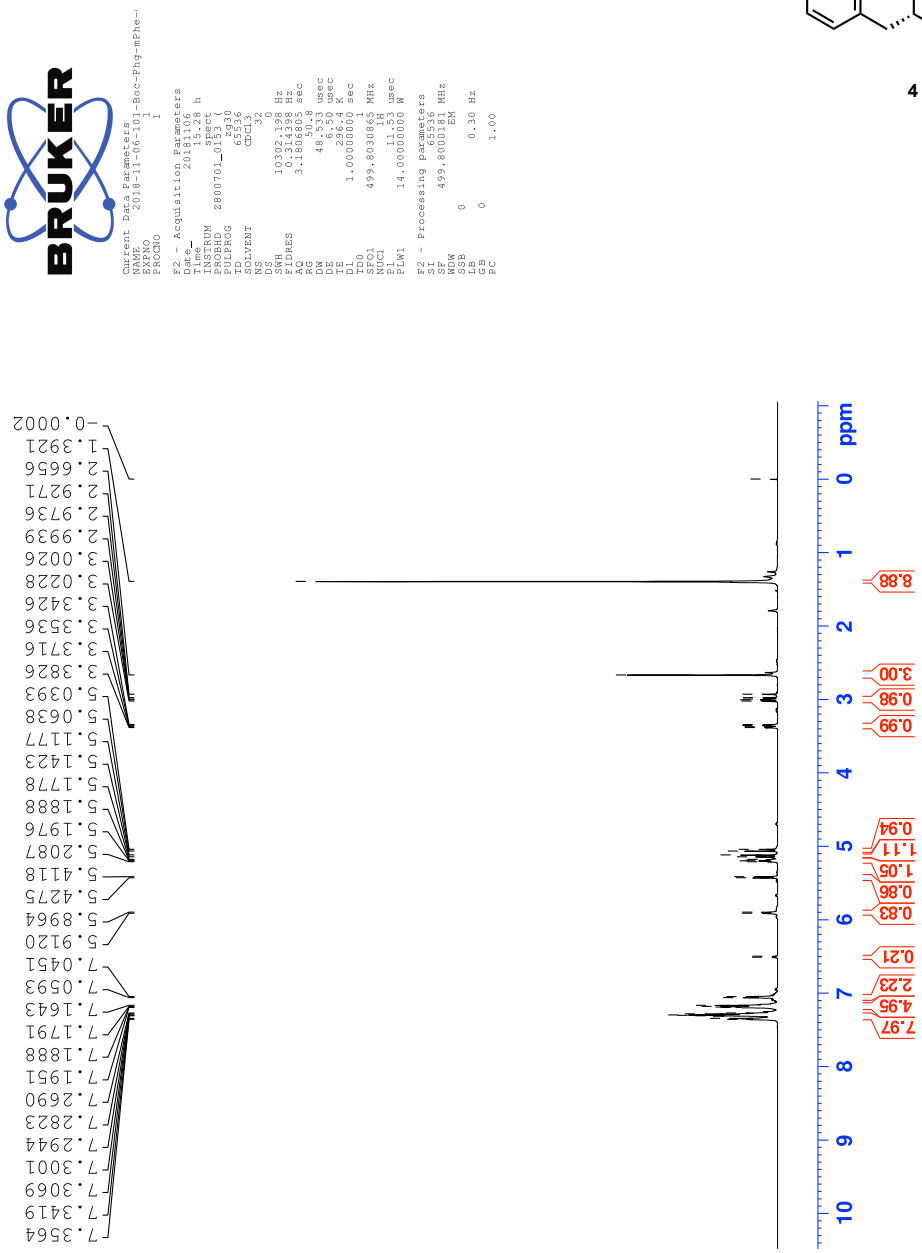
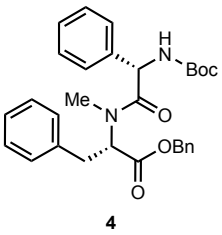


Compound 4: ^{1}H-NMR



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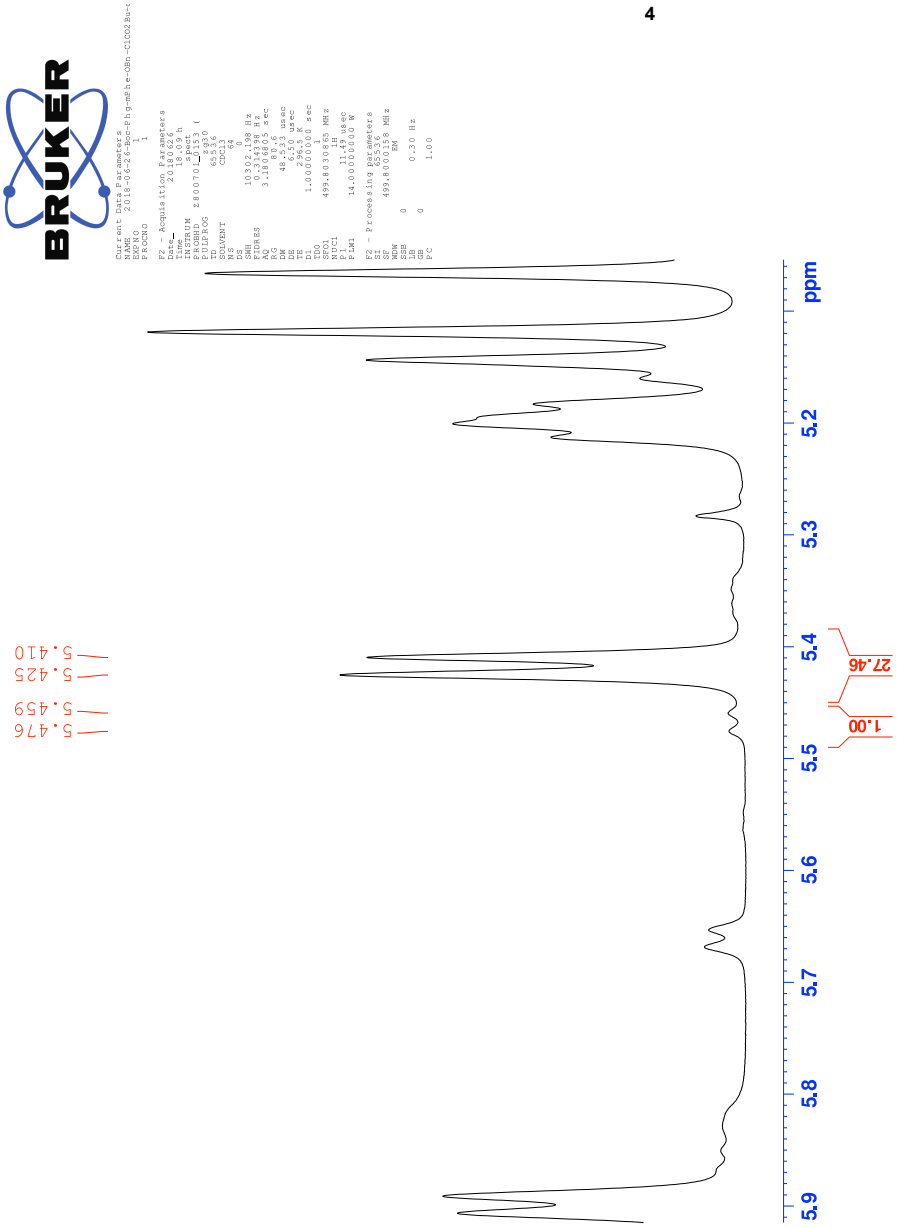
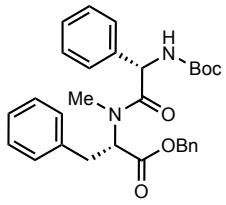


Compound 4: ^{13}C-NMR



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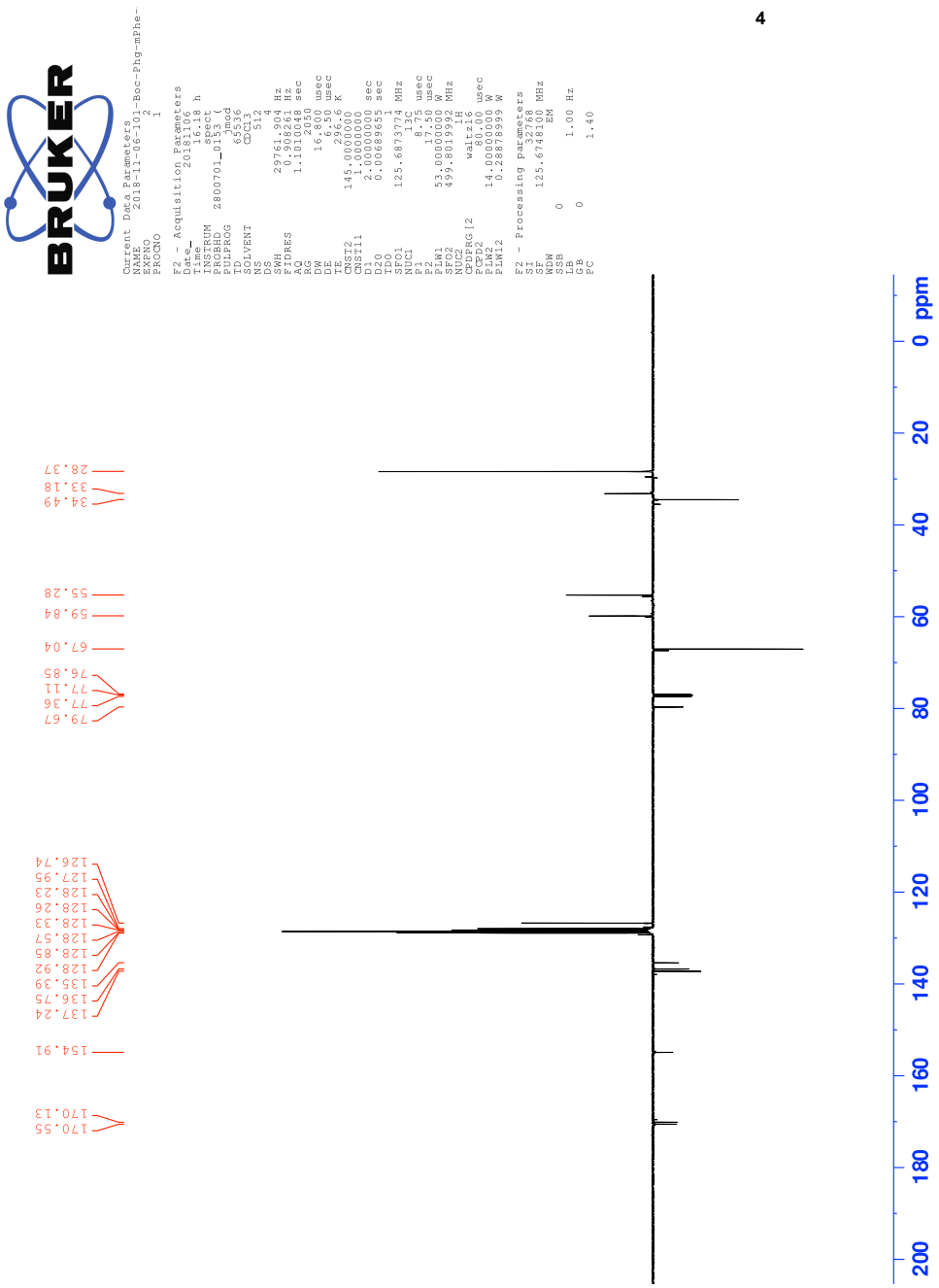
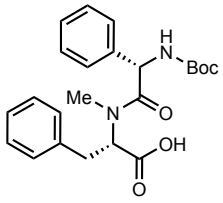


Compound 7: ^{1}H-NMR



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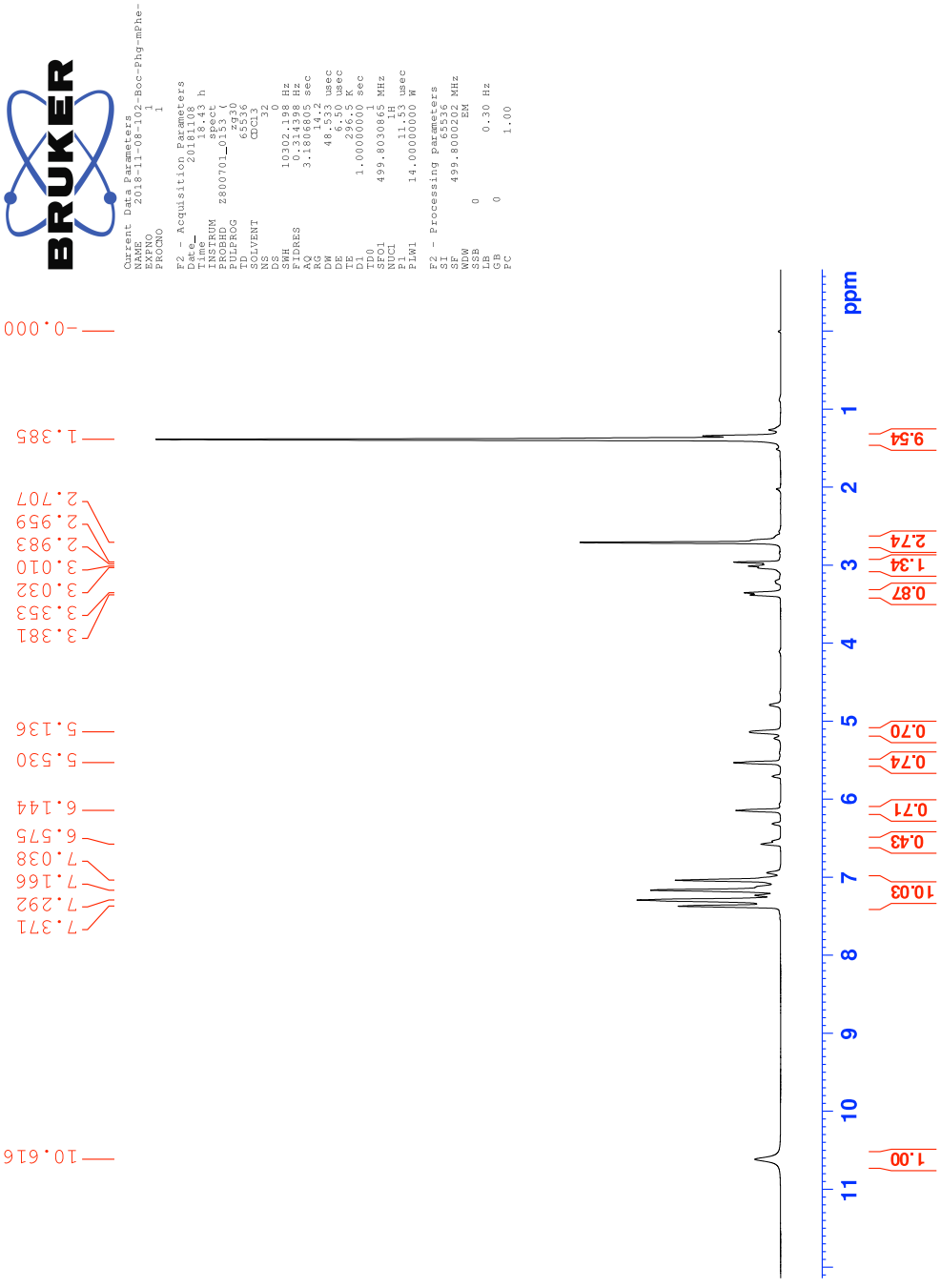
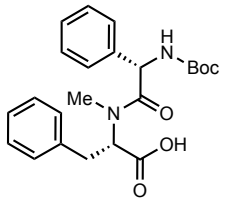


Compound 7: ^{13}C-NMR



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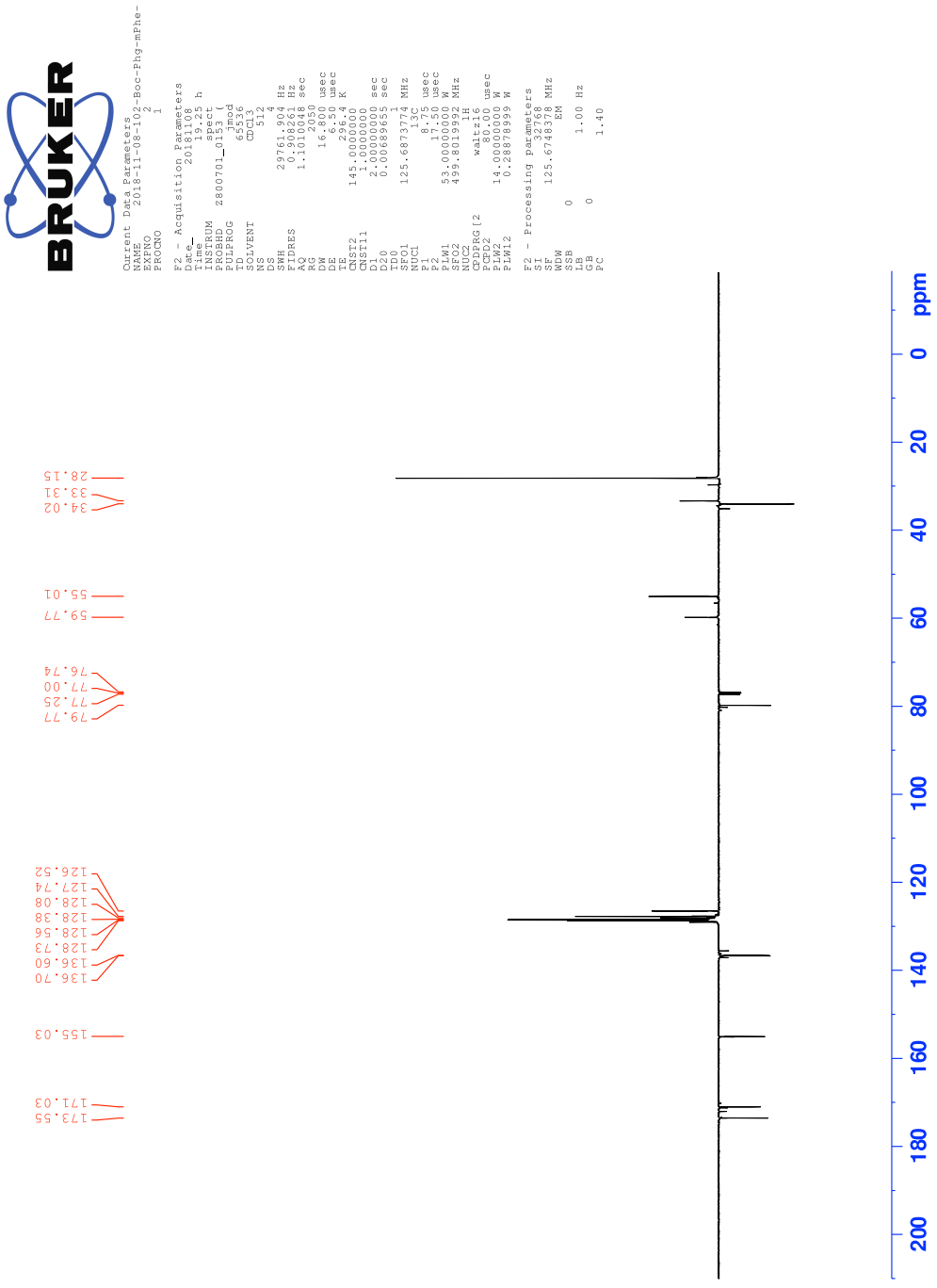
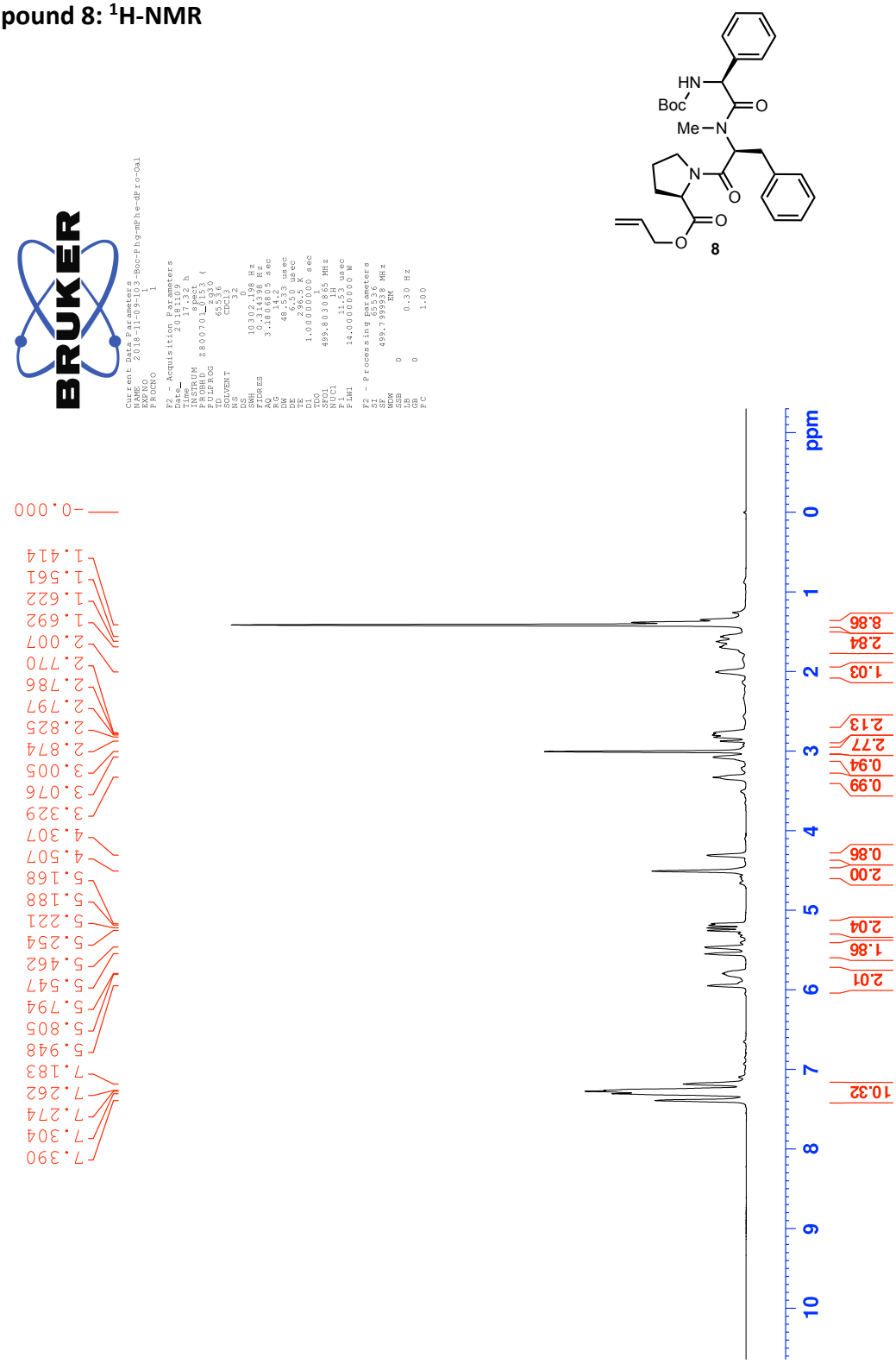
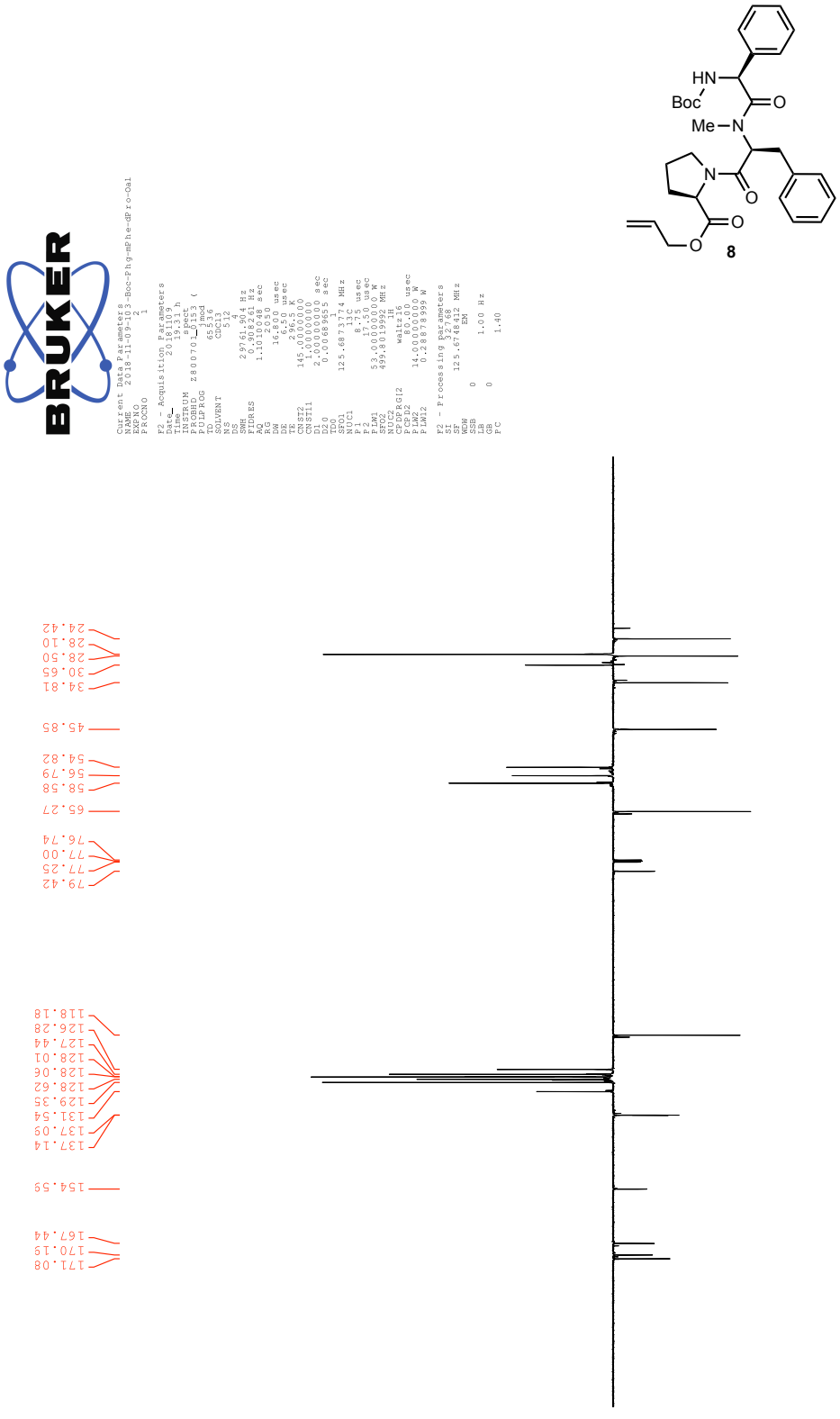


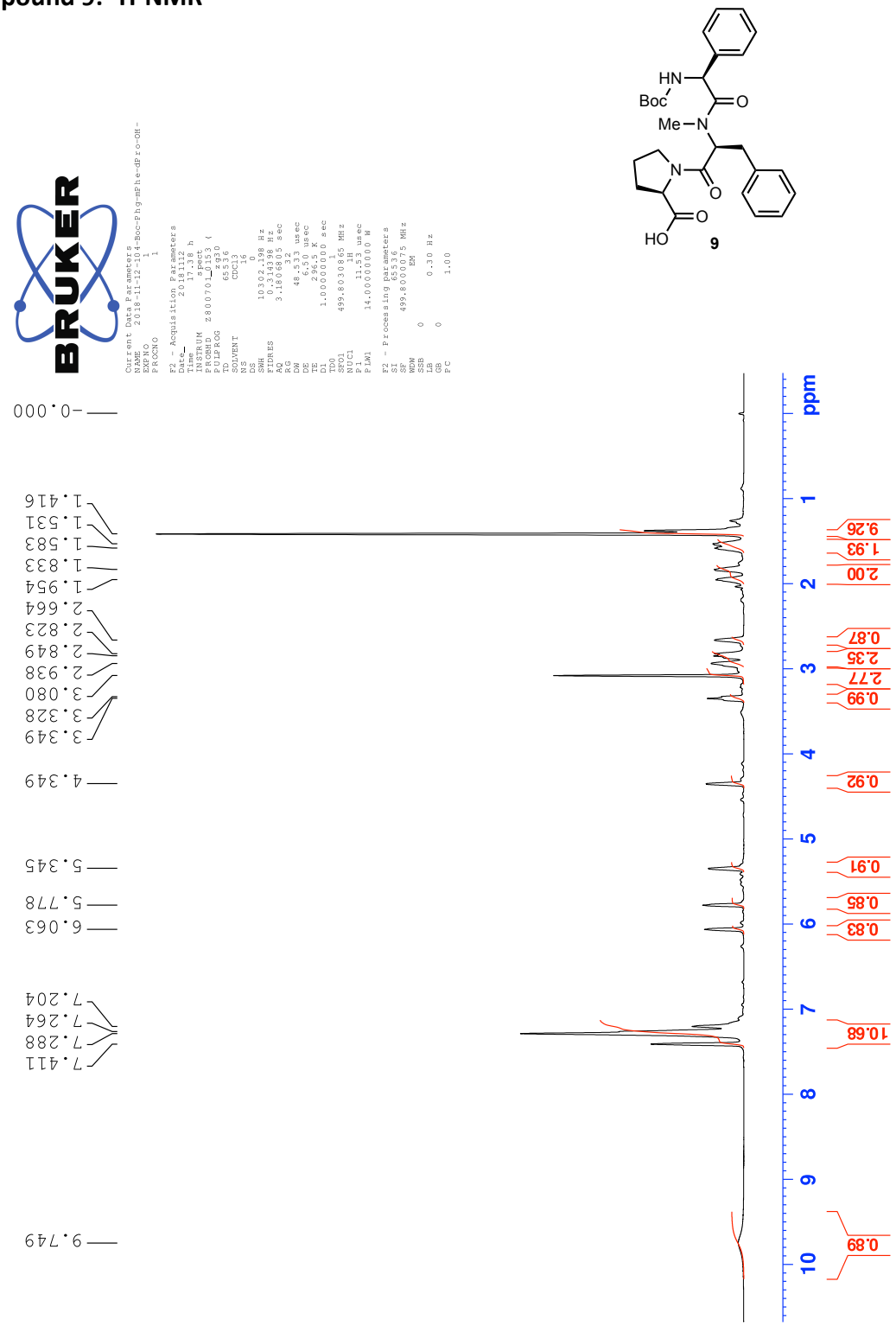
Compound 8: ^{1}H-NMR



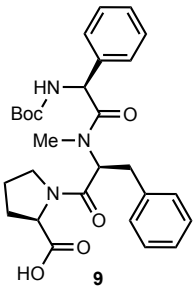
Compound 8: ^{13}C-NMR

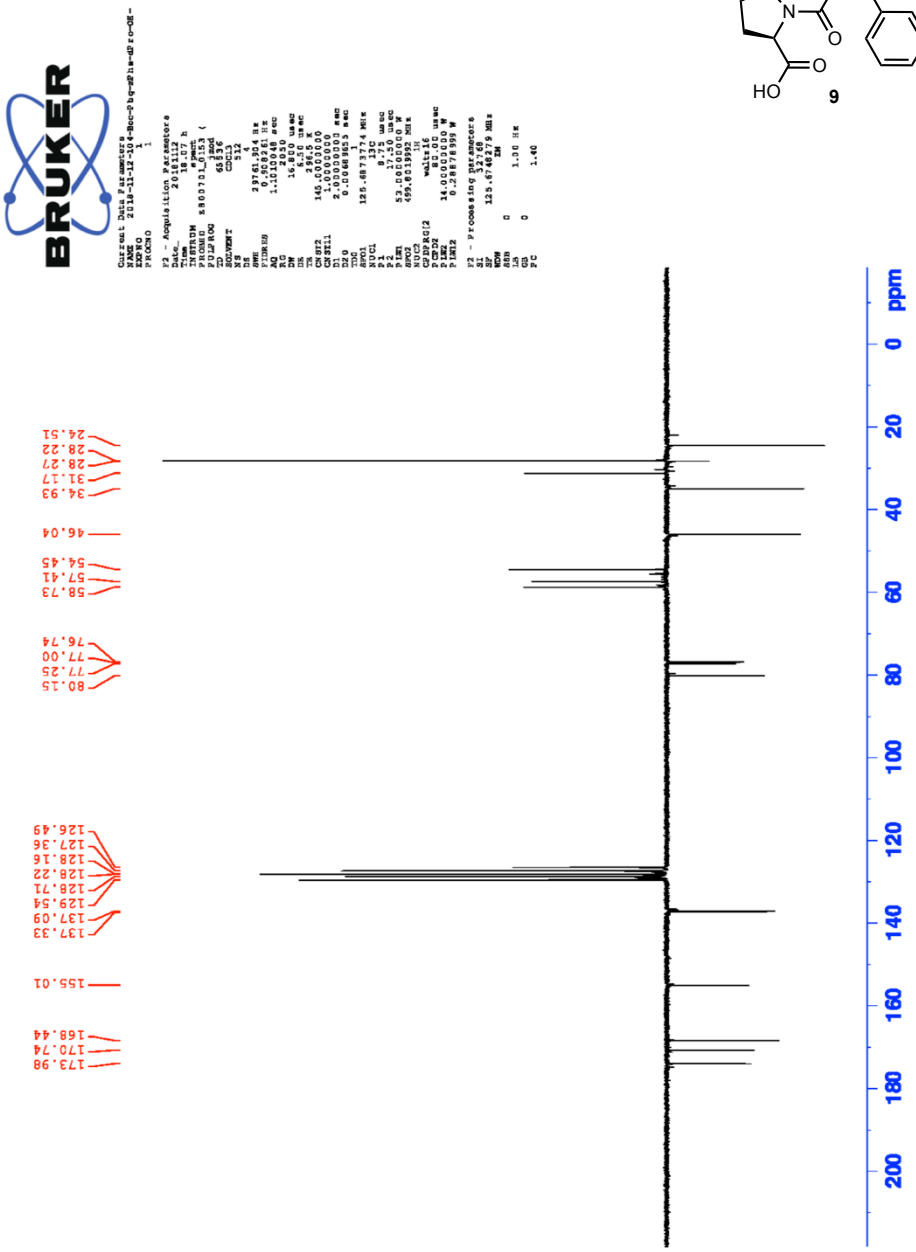


Compound 9: ^{1}H-NMR

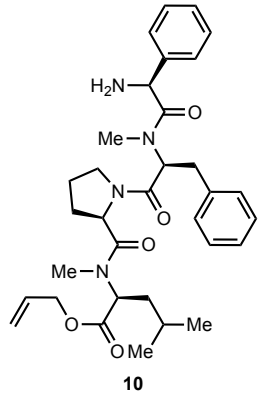


Compound 9: ^{13}C-NMR





Compound 10: ^{1}H-NMR



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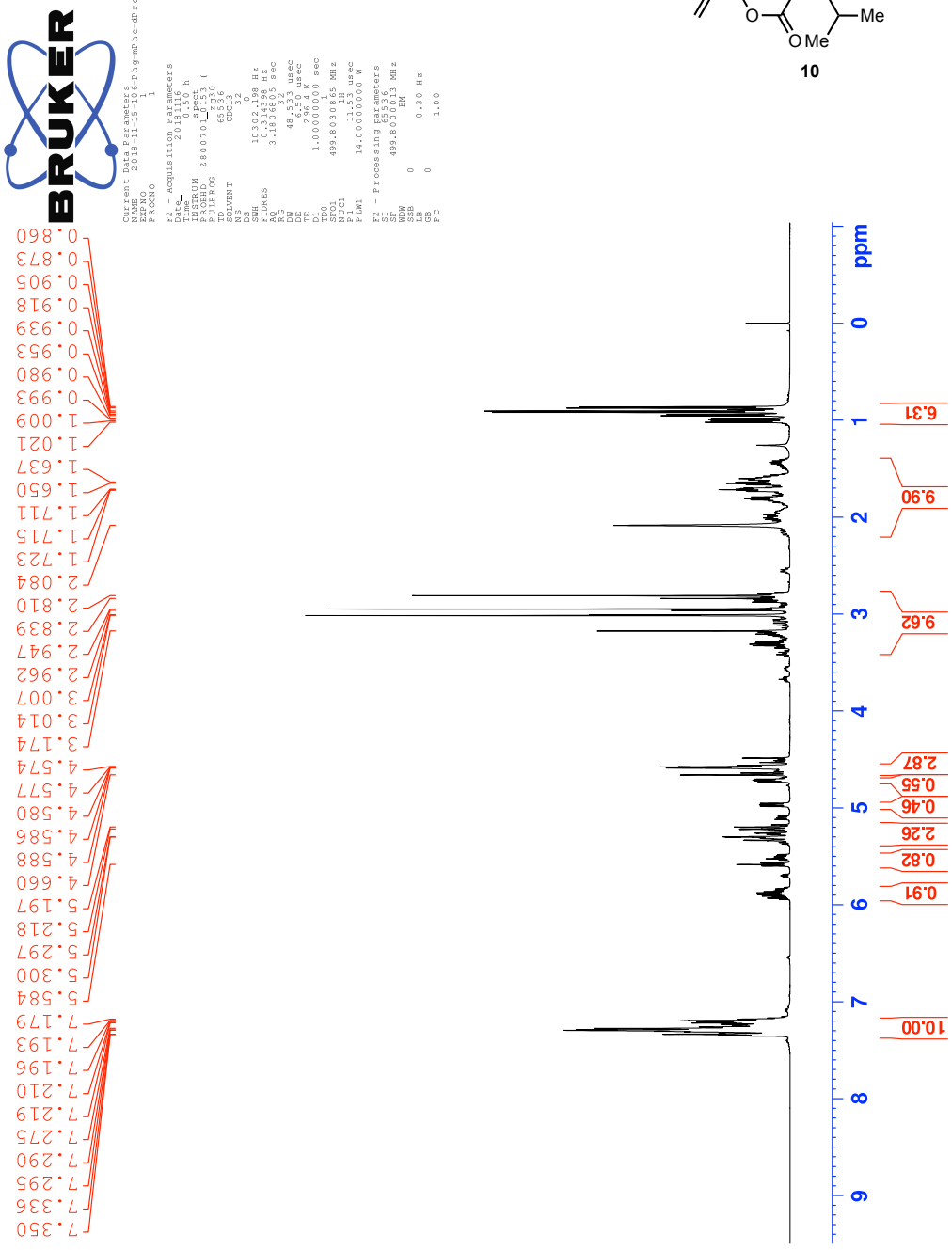
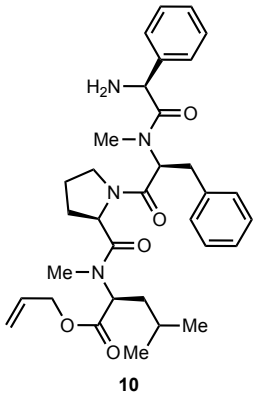


Compound 10: ^{13}C-NMR



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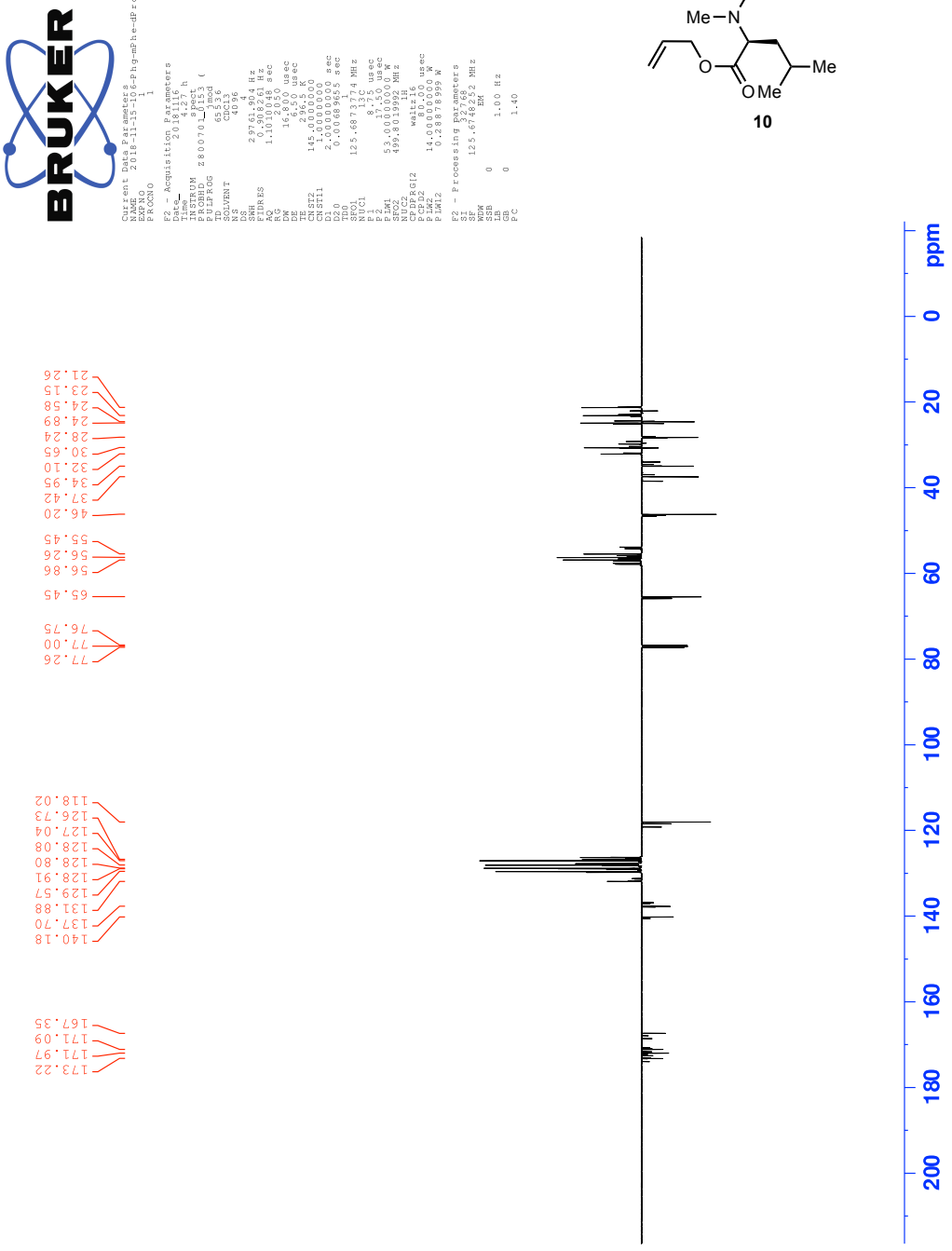
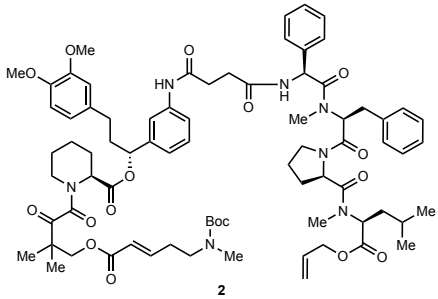
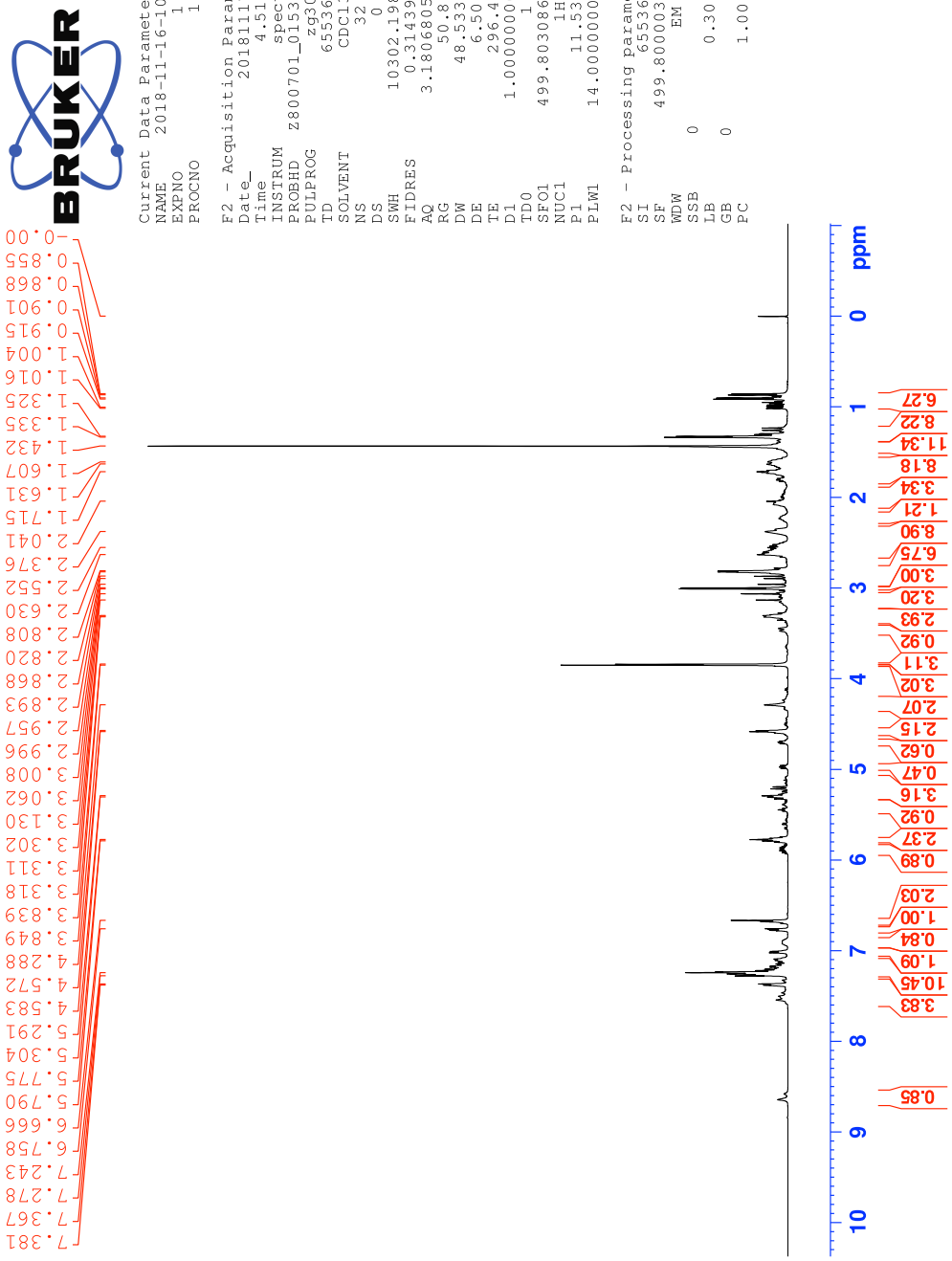


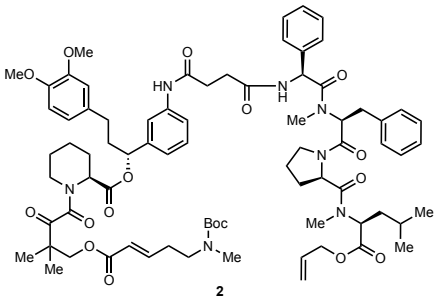
Compound 2: ^{1}H-NMR

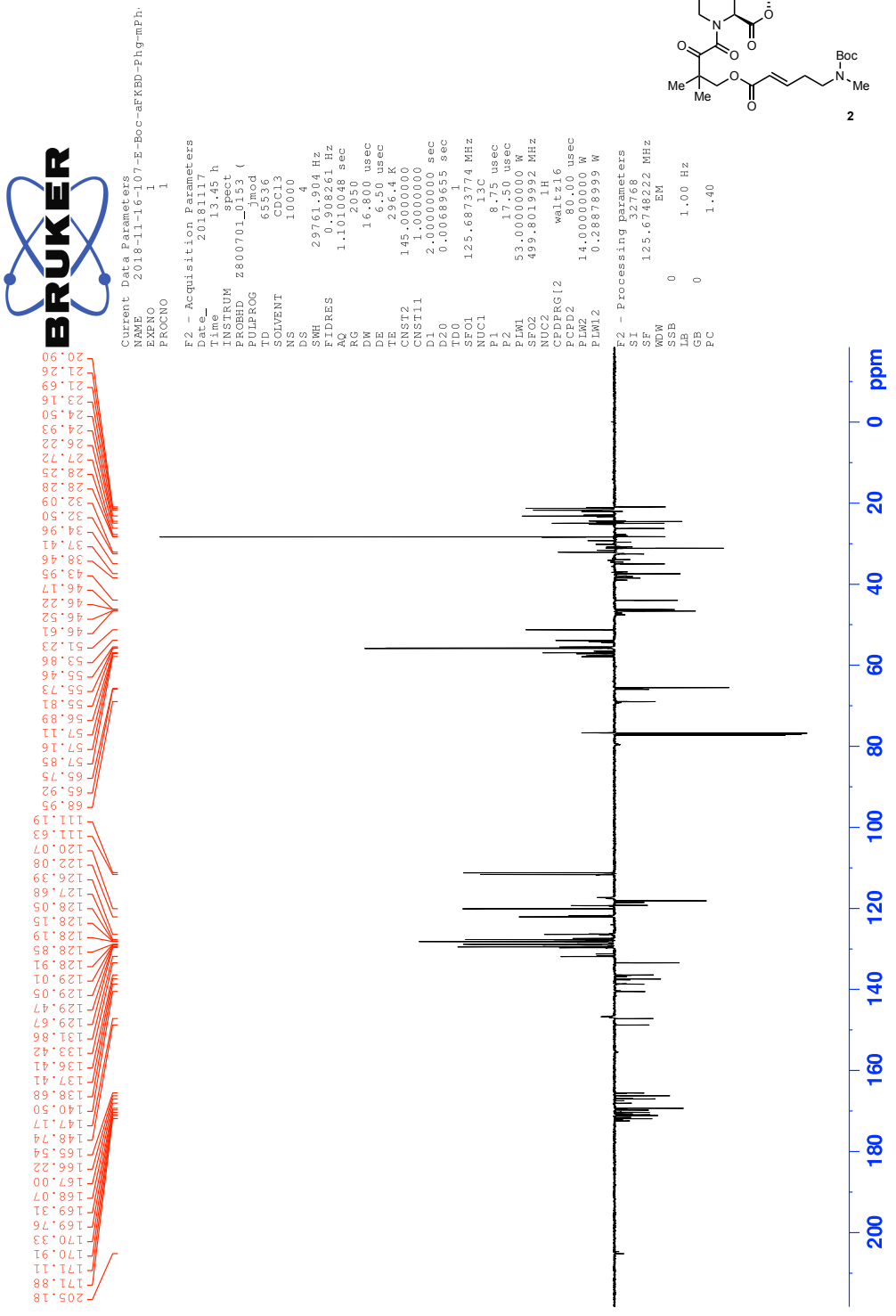


2

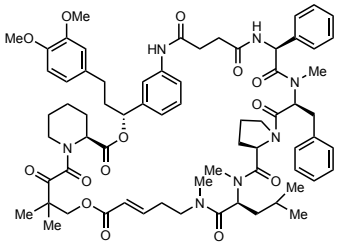


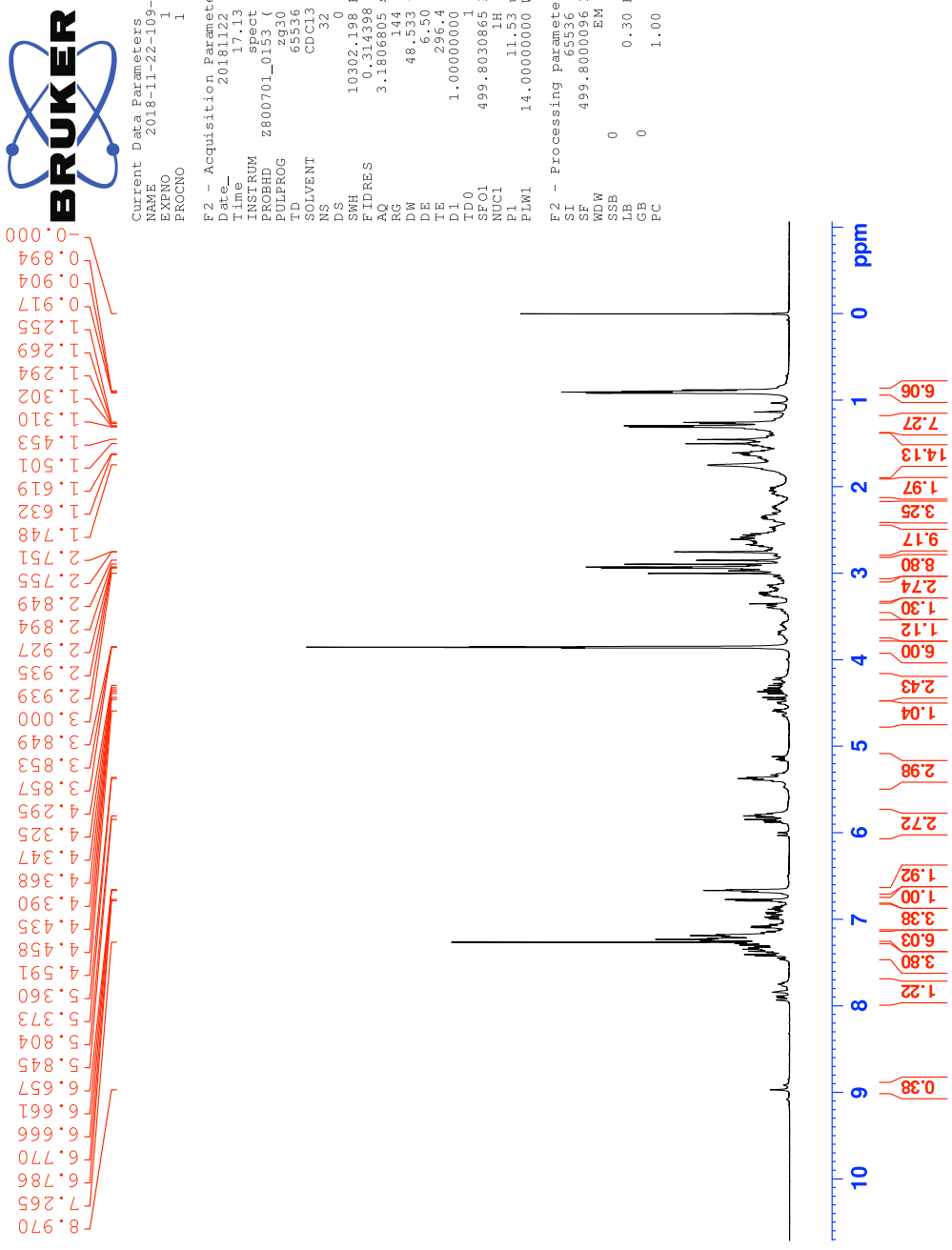
Compound 2: ^{13}C-NMR



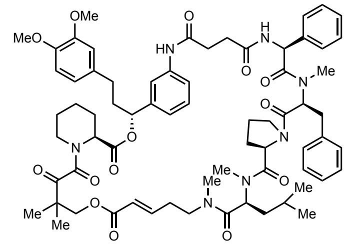


Rapadocin 1a: ^{1}H-NMR

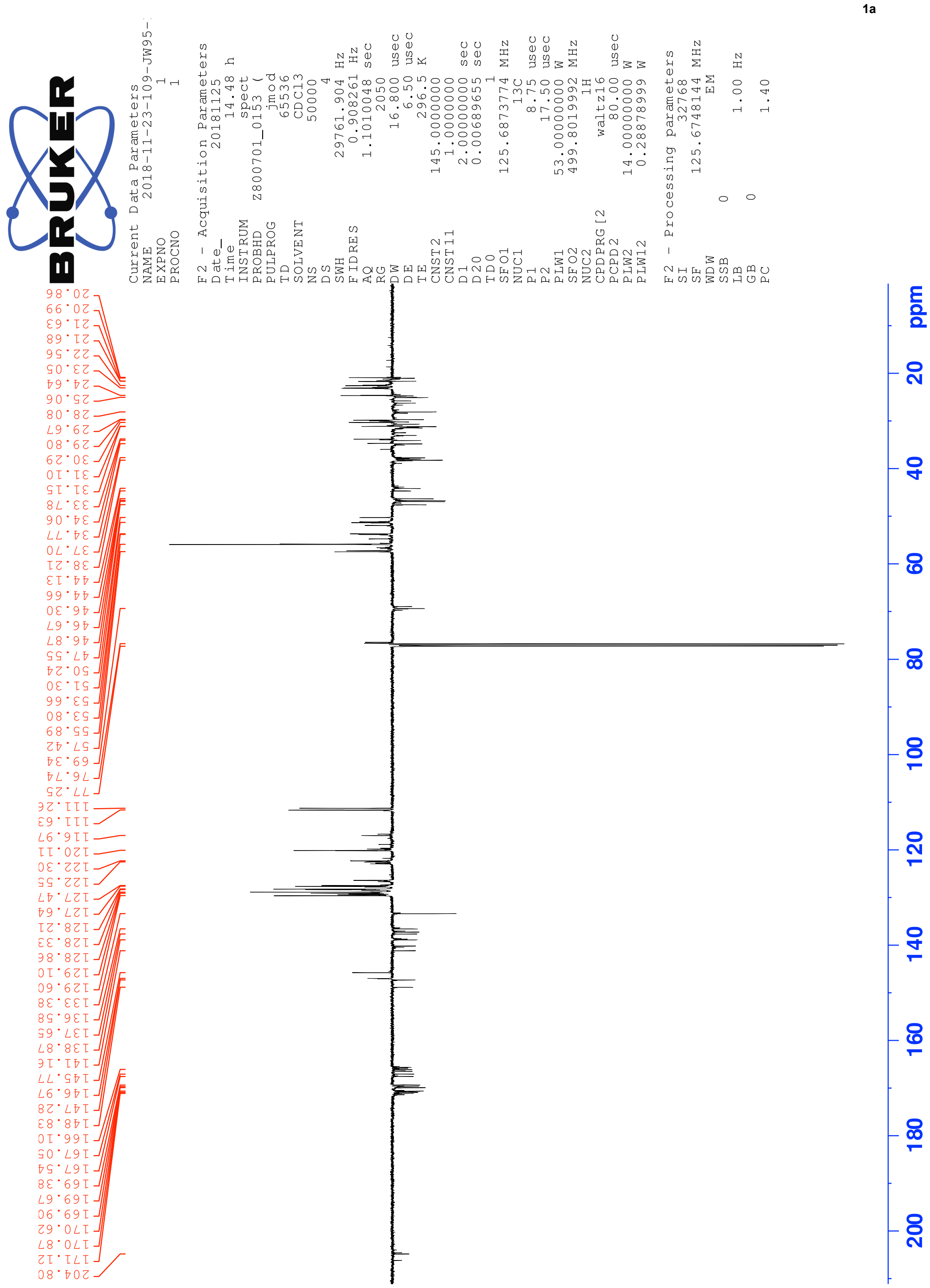




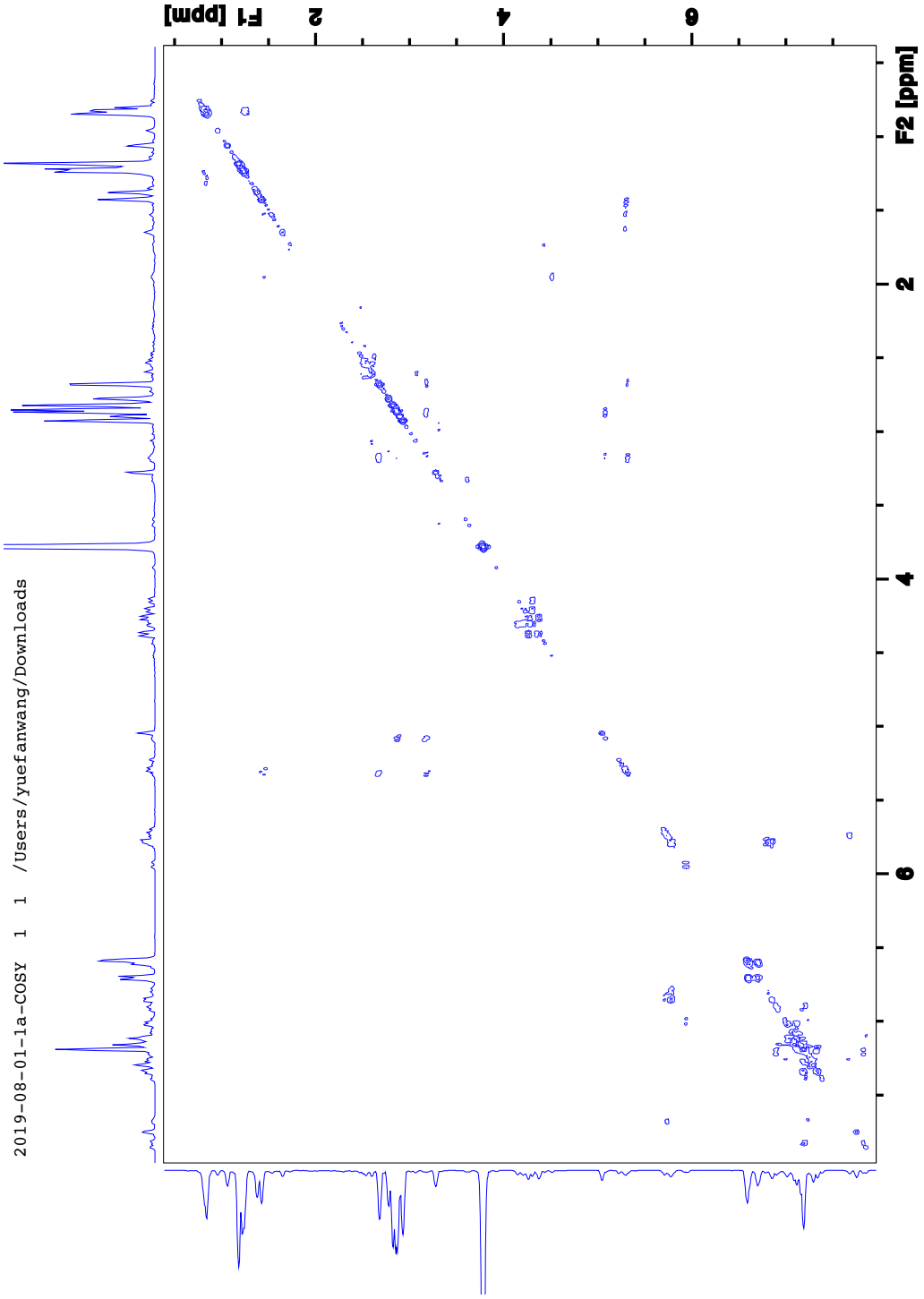
Rapadocin 1a: ^{13}C-NMR



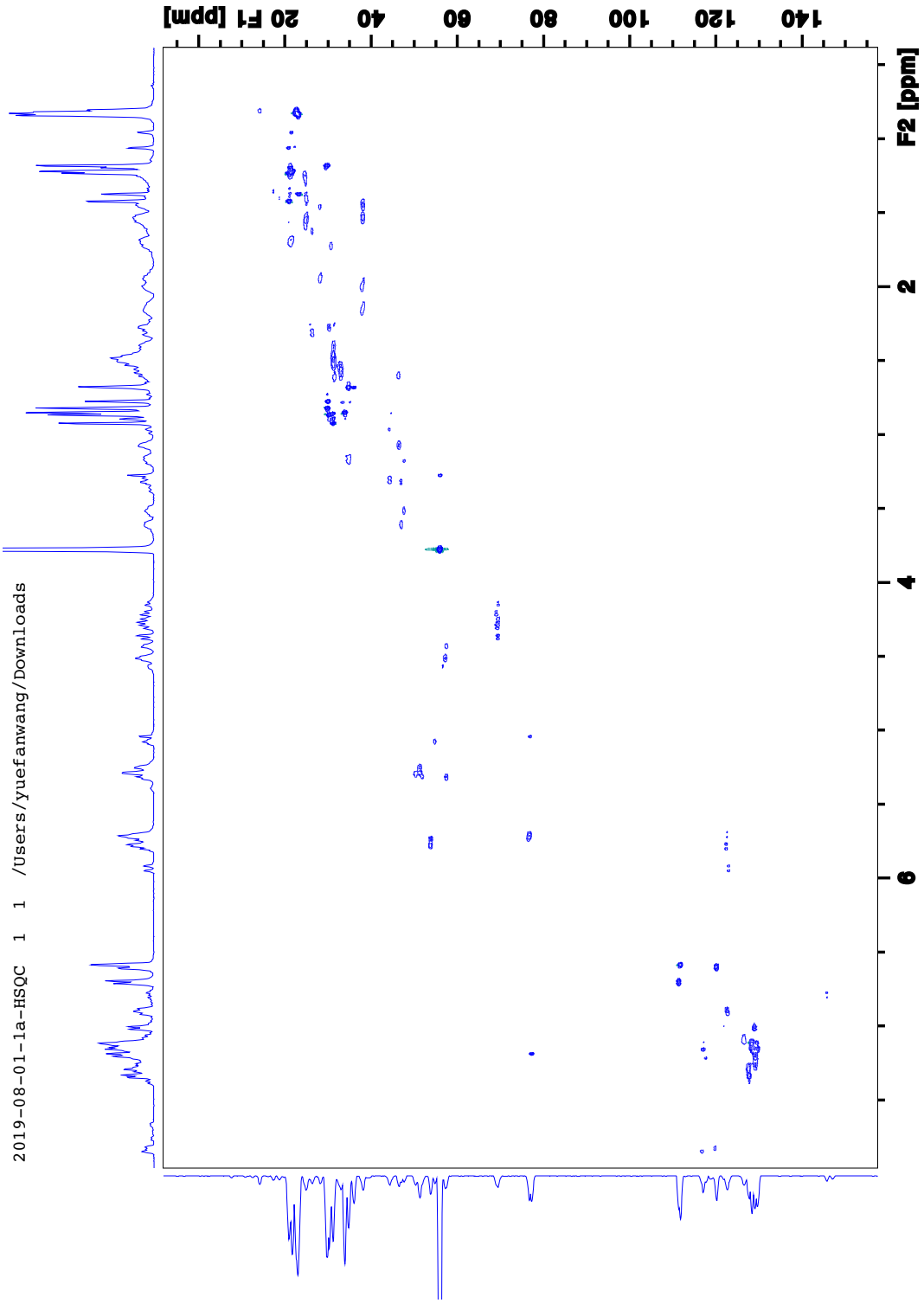
1a



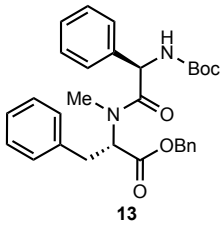
Rapadocin 1a: ^{1}H-^{1}H COSY



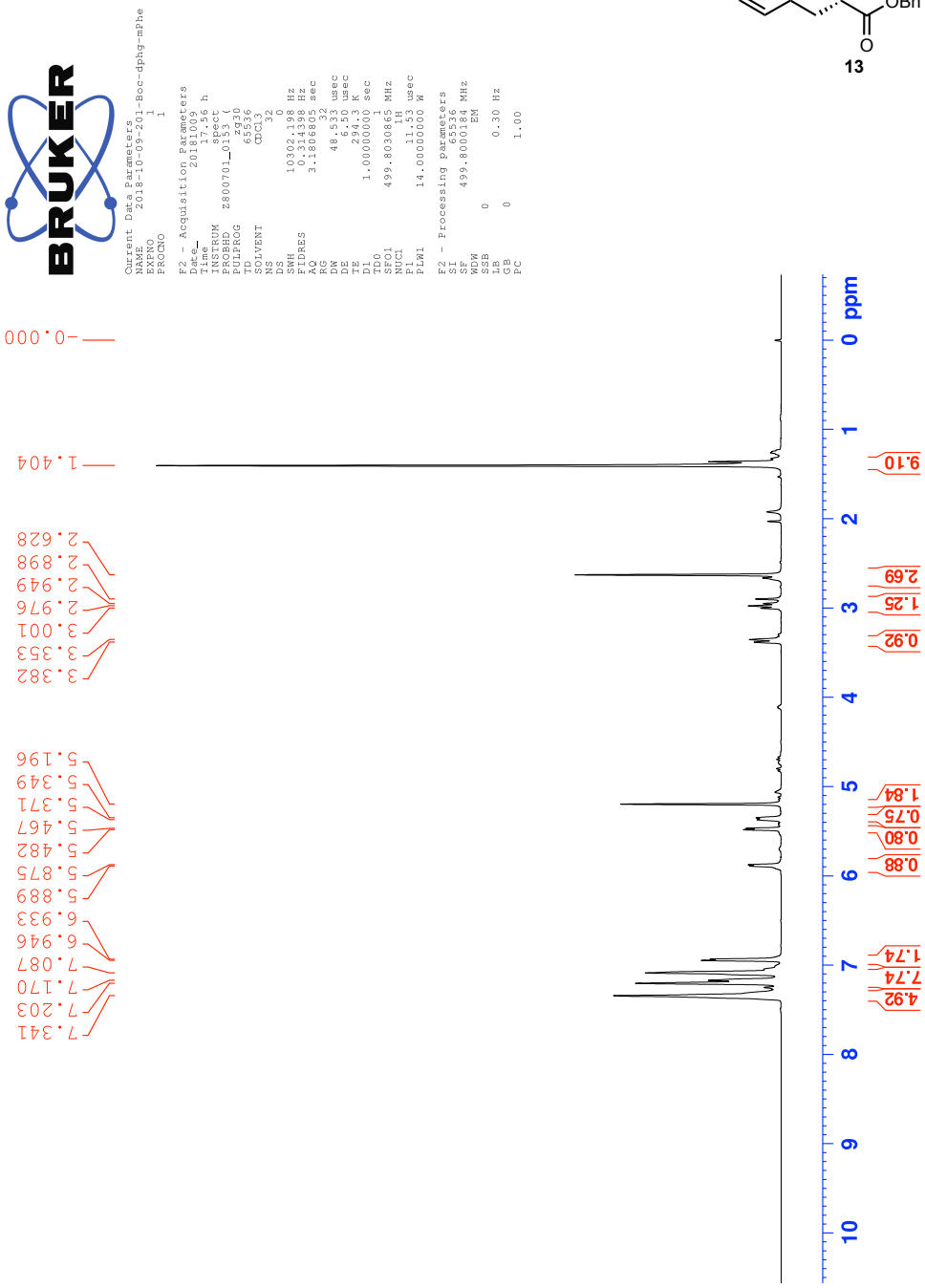
Rapadocin 1a: ^{1}H-^{13}C HSQC



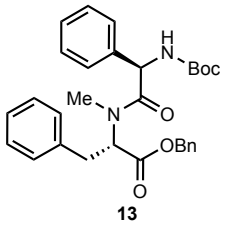
Compound 13: ^{1}H-NMR

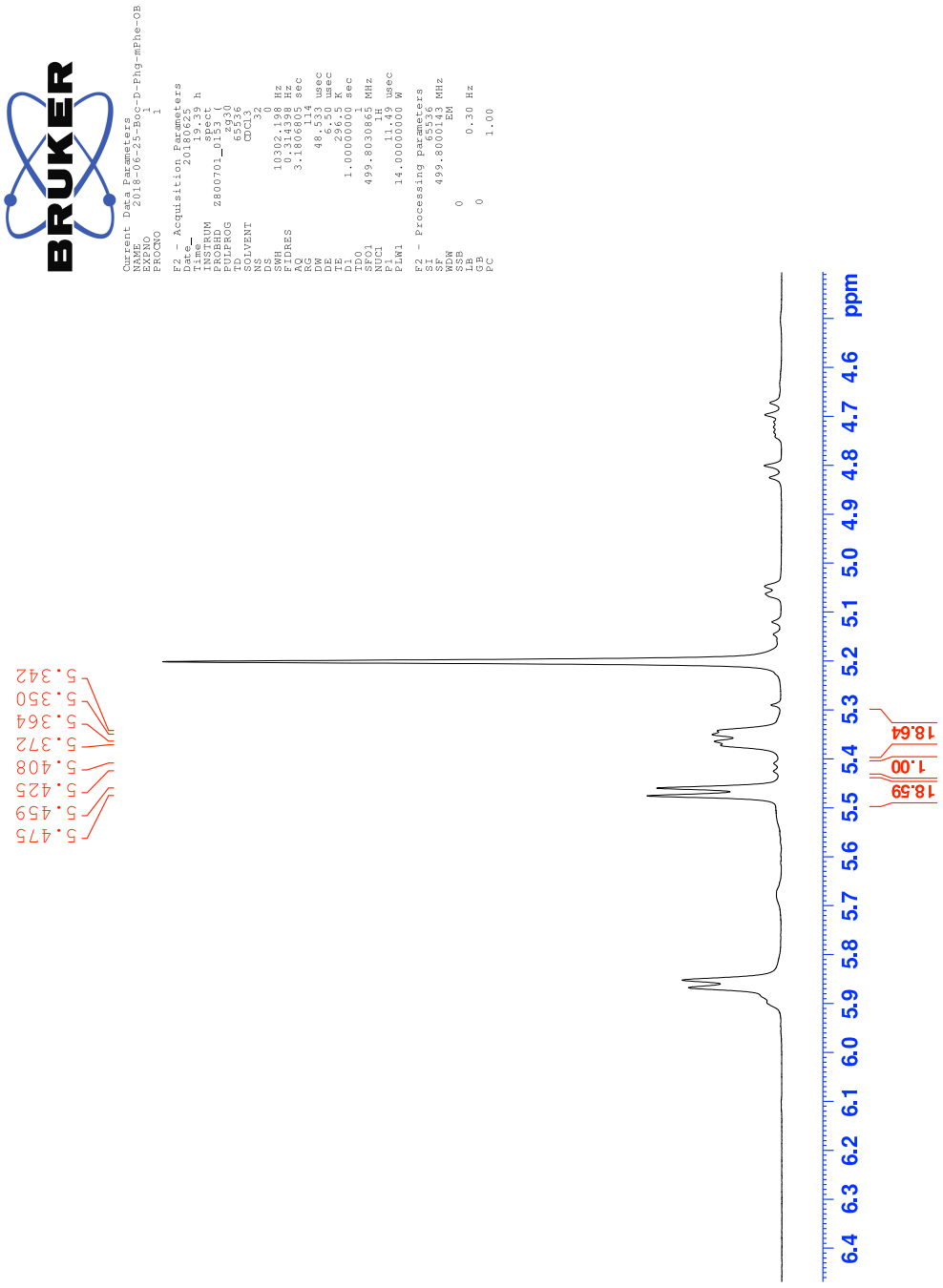


13

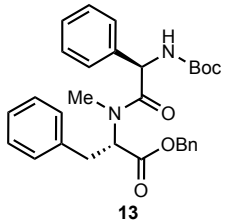


Compound 13: ^{1}H-NMR





Compound 13: ^{13}C-NMR



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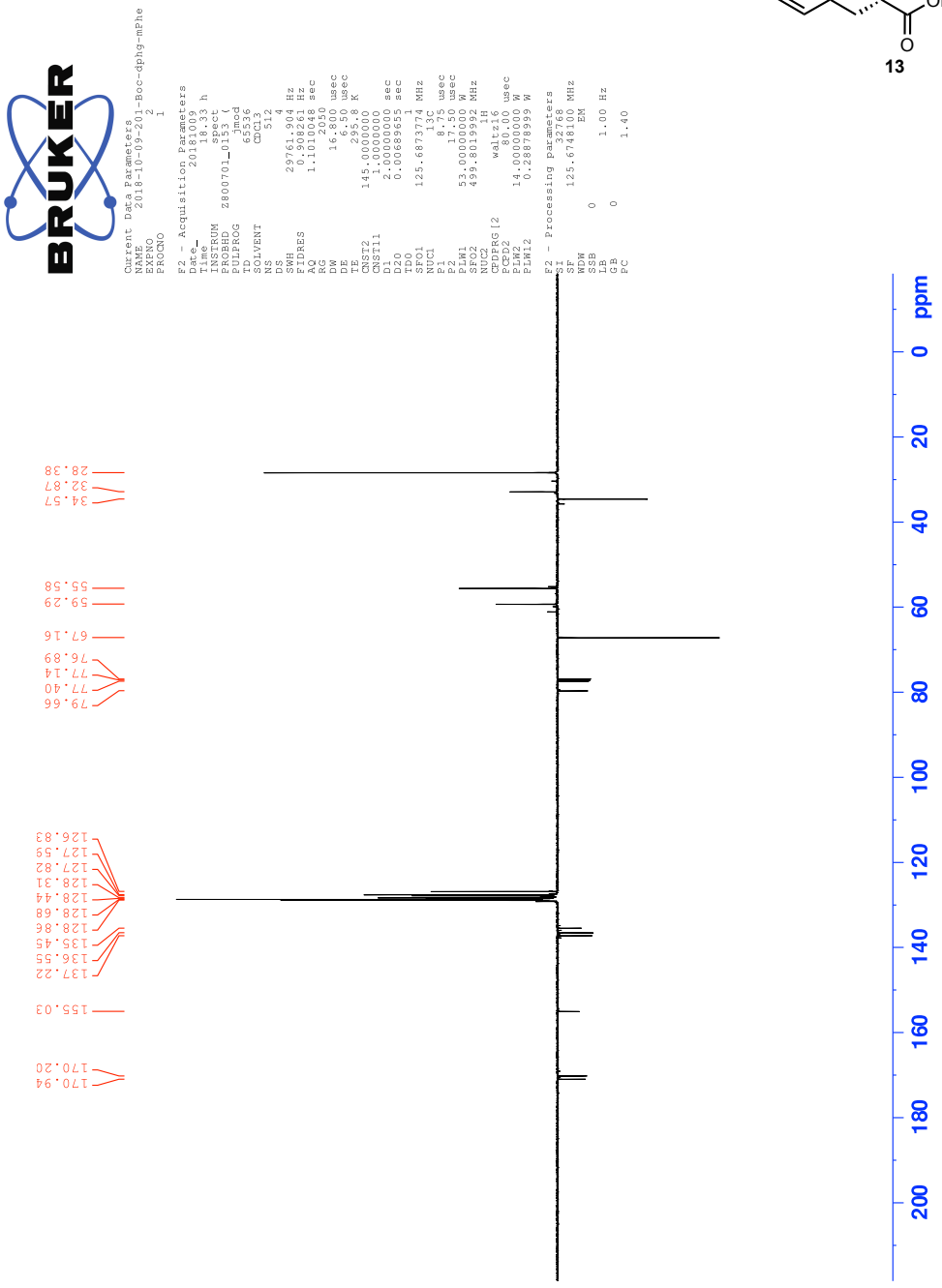
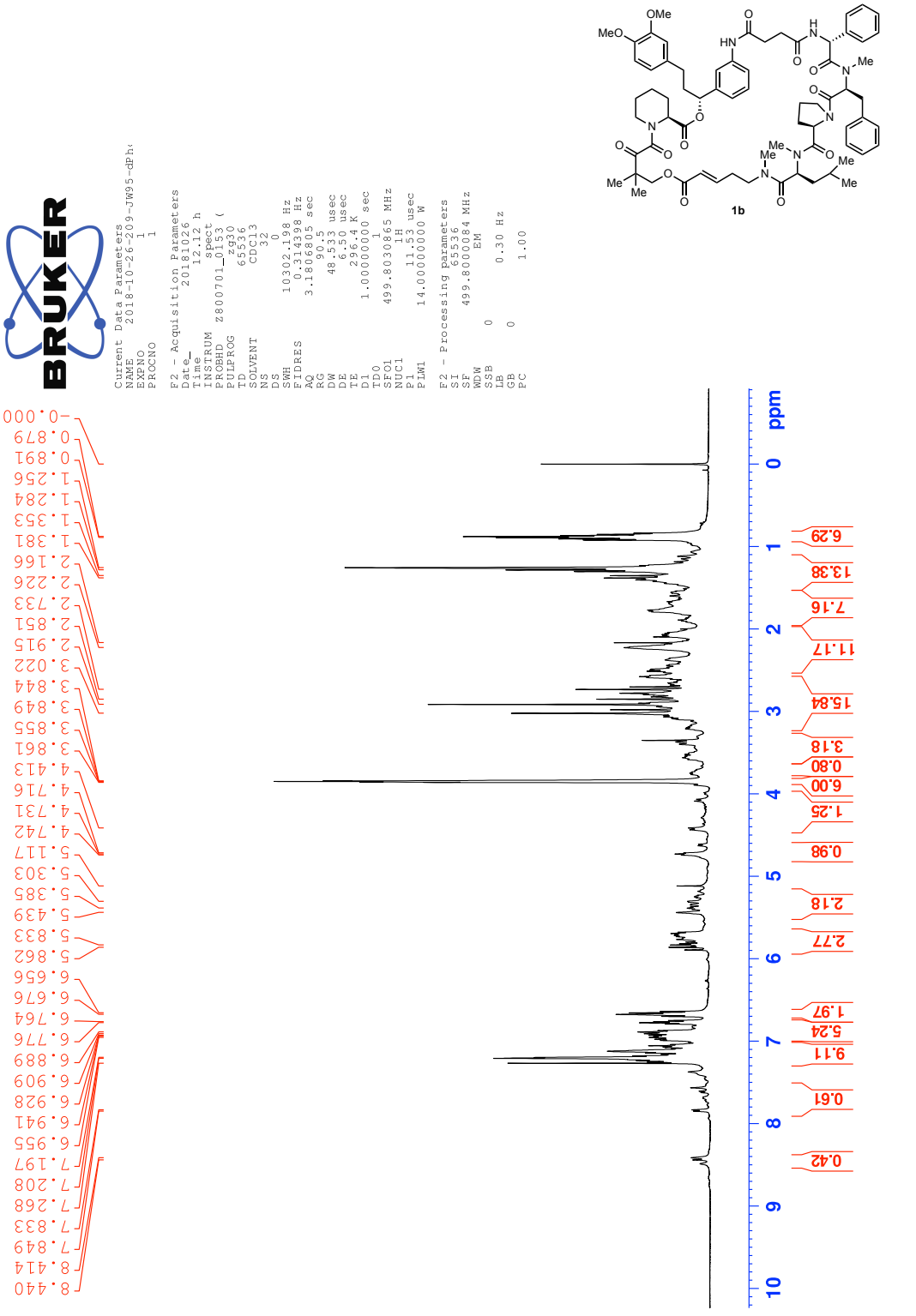
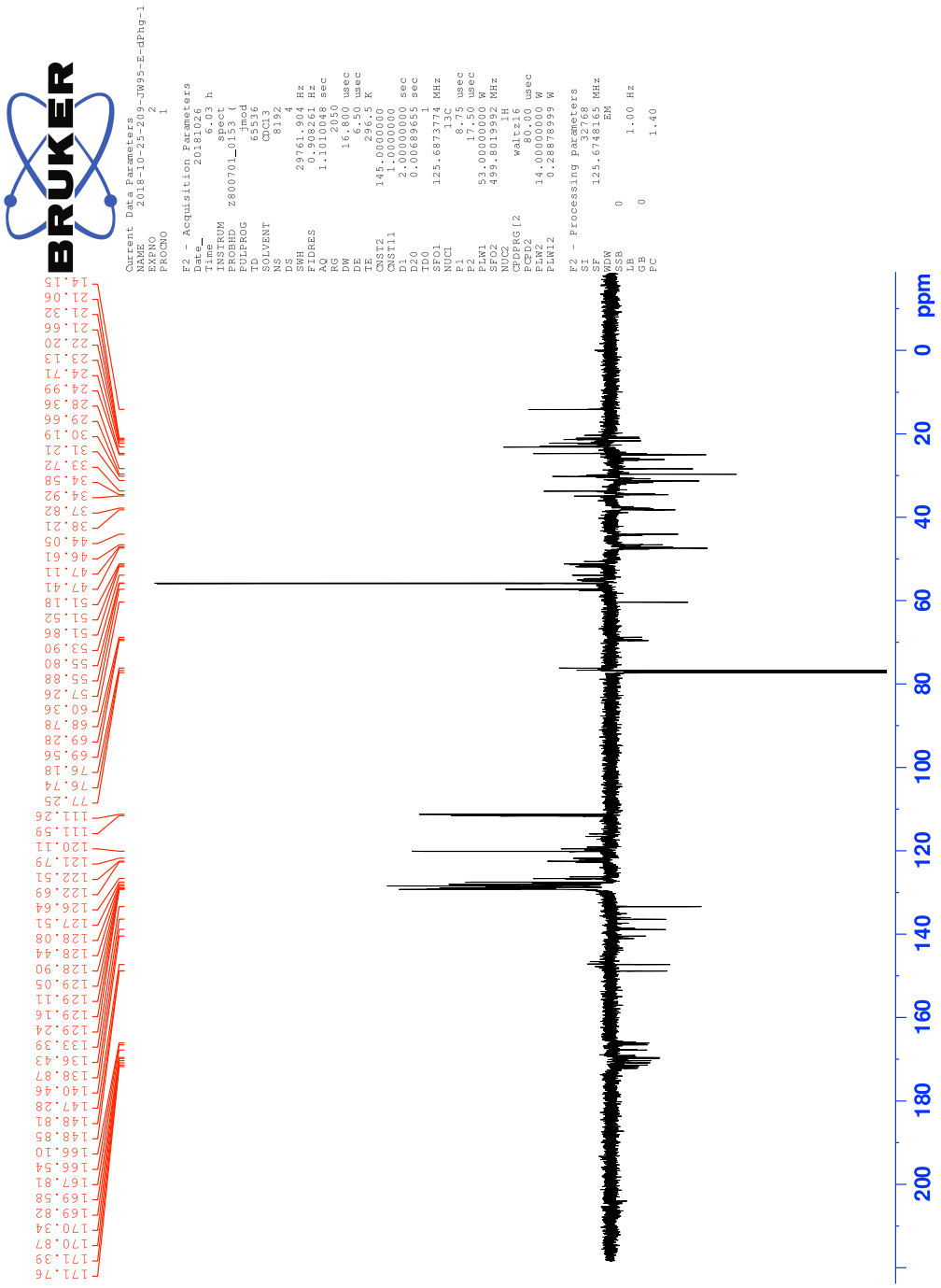


Rapadocin 1b: ^{1}H-NMR

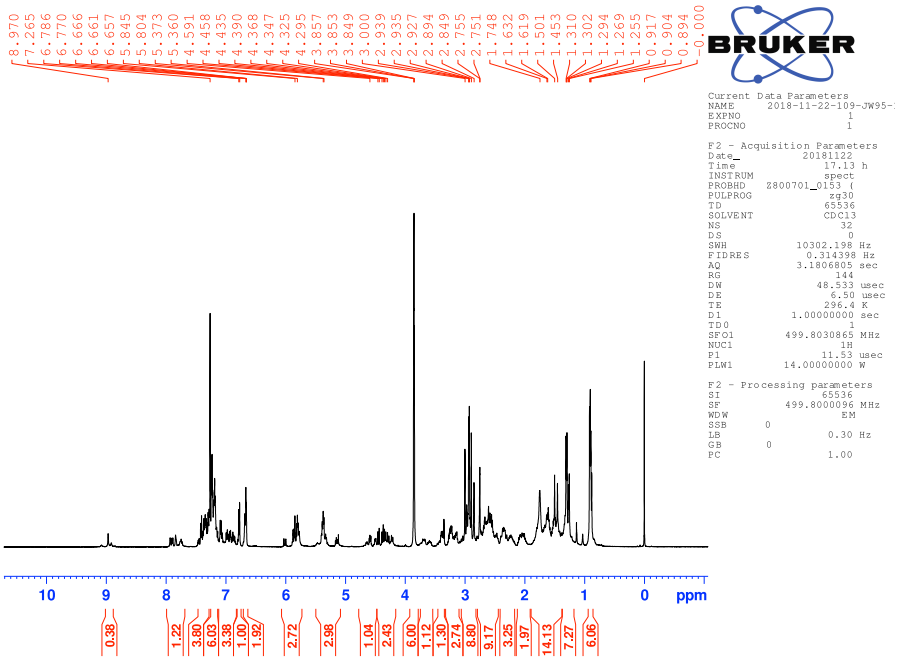


Rapadocin 1b: ^{13}C-NMR

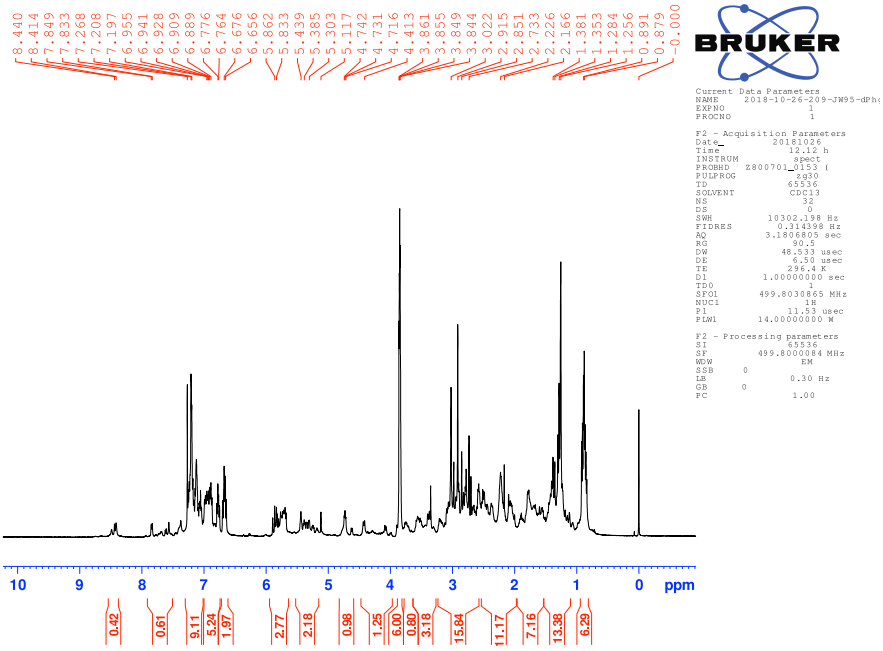


Comparison of the Spectra of Rapadocin isomers and Solid Phase Products

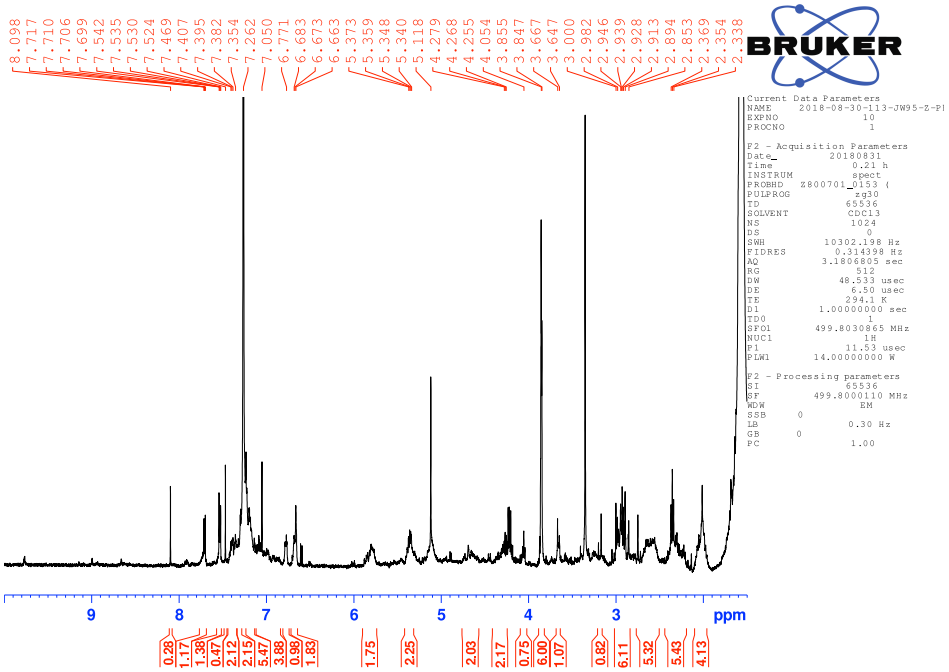
Rapadocin **1a**



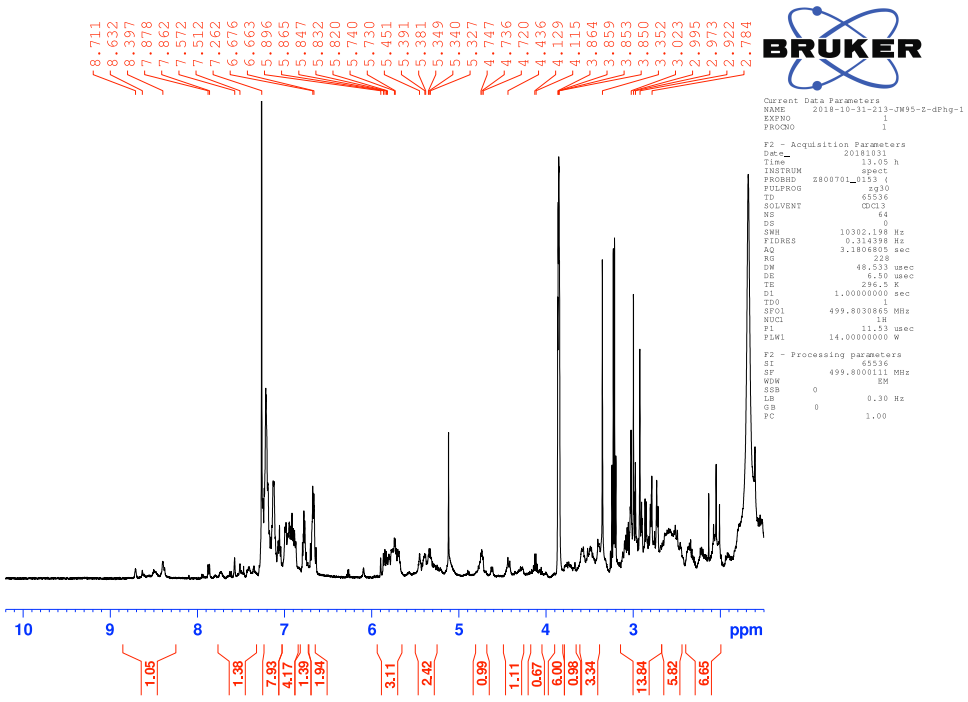
Rapadocin **1b**



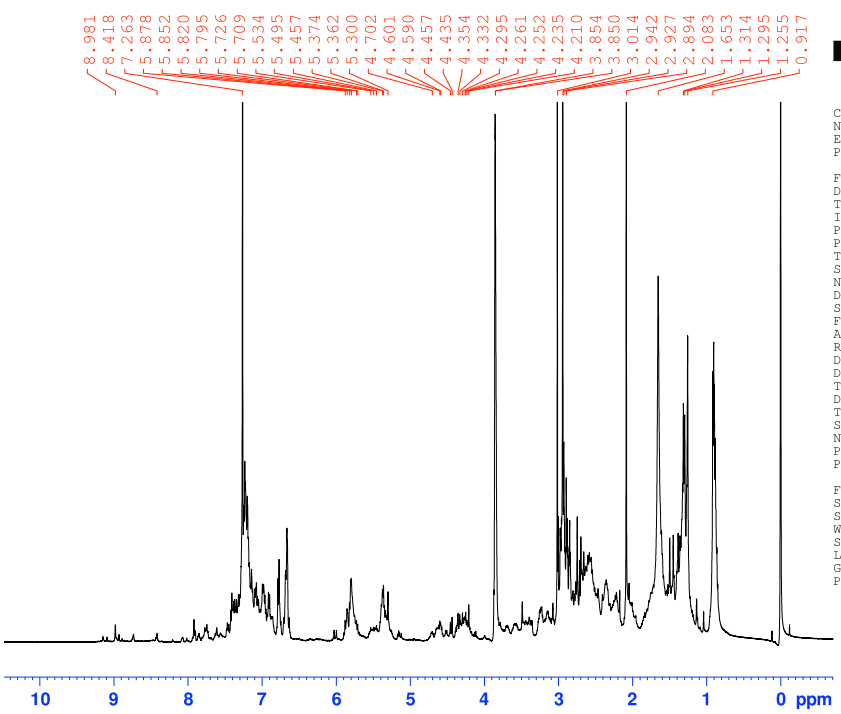
Rapadocin **1c**



Rapadocin **1d**



Rapadocin from solid phase synthesis



BRUKER

Current Data Parameters
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PROCNO 1

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INSTRUM spect
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SOLVENT CDCl3
NS 256
DS 0
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FIDRES 0.314398 Hz
AQ 3.1806805 sec
RG 203
DW 48.533 usec
DE 6.50 usec
TE 296.5 K
D1 1.00000000 sec
TD0 1
SFO1 499.8030865 MHz
NUC1 1H
P1 11.53 usec
PLW1 14.00000000 W

F2 - Processing parameters
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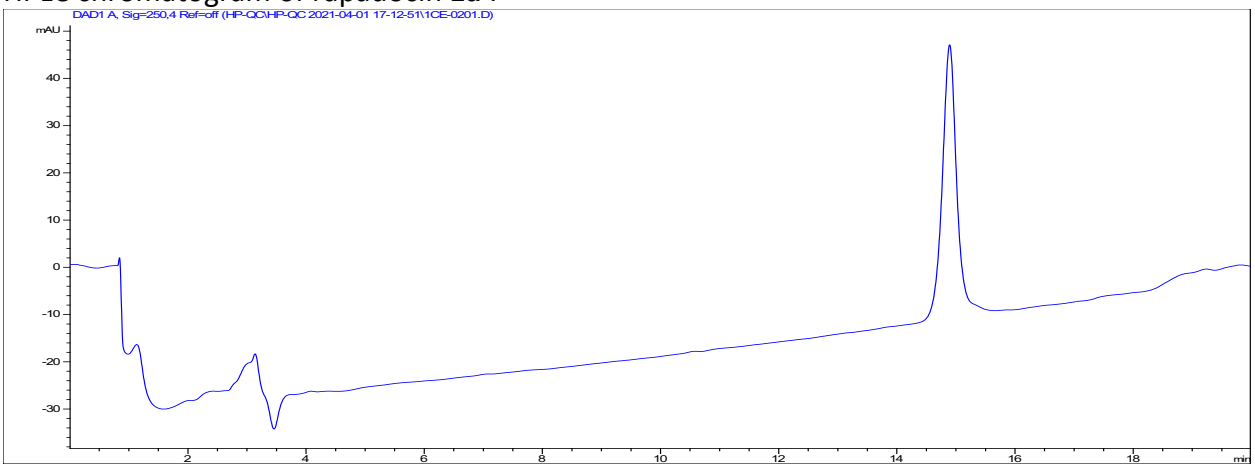
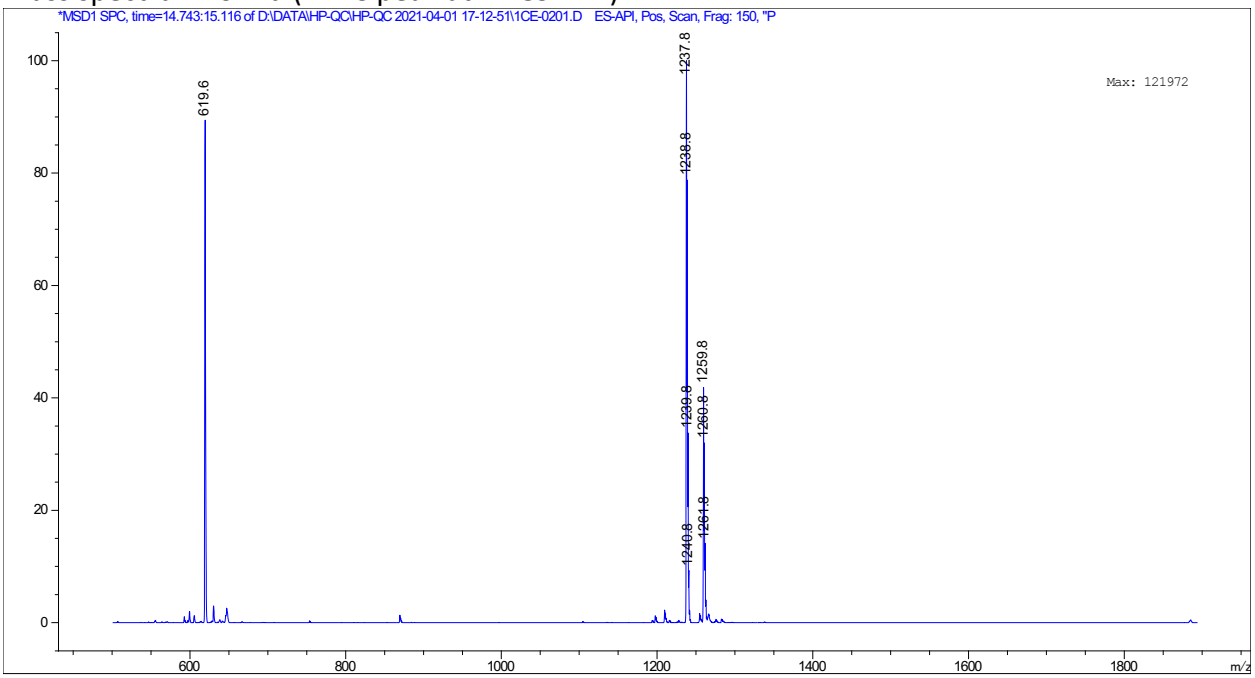
SF 499.8000101 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

Chromatogram of HPLC of the Rapadocin isomers and MS of the HPLC peak

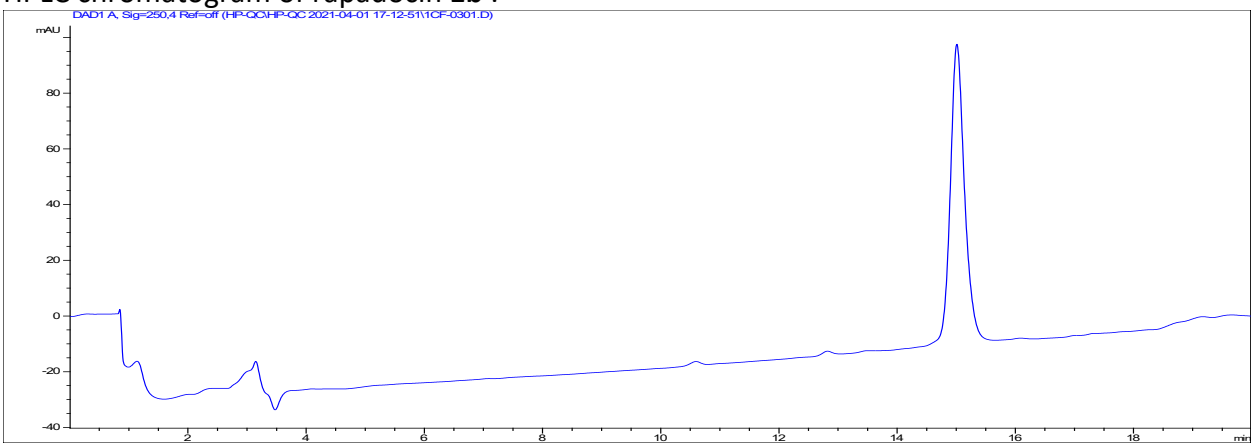
HPLC chromatogram of rapadocin **1a** :



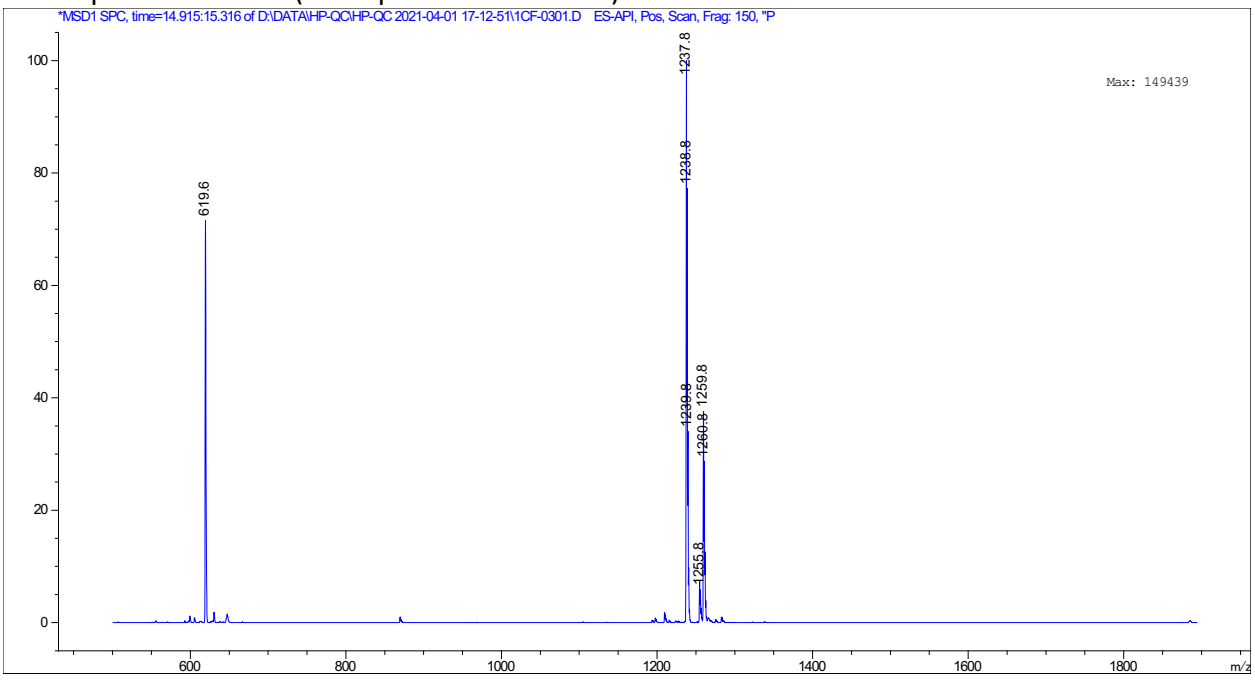
Mass spectrum for **1a** (HPLC peak at 14.89 min):



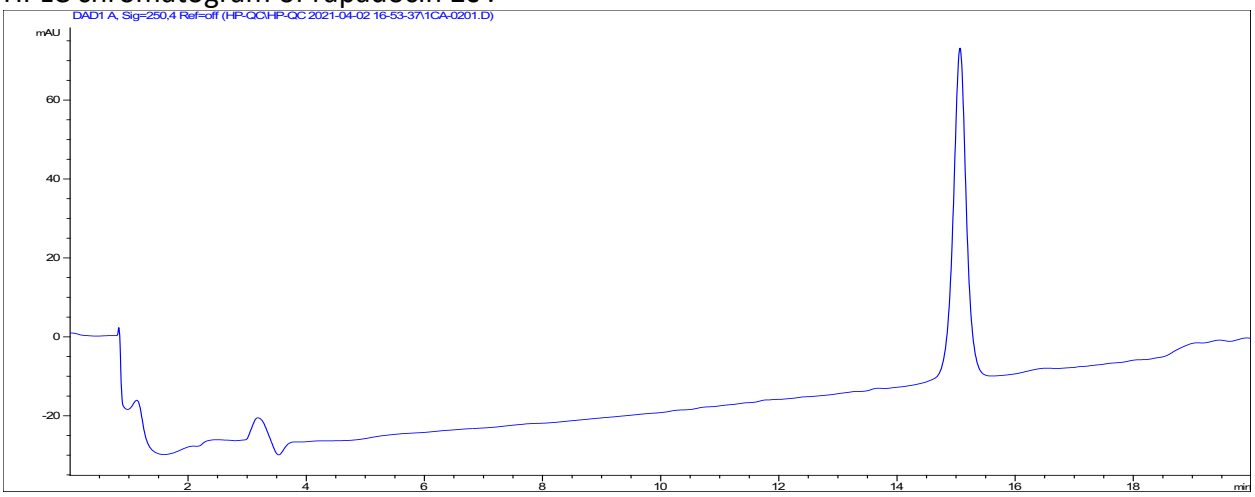
HPLC chromatogram of rapadocin **1b** :



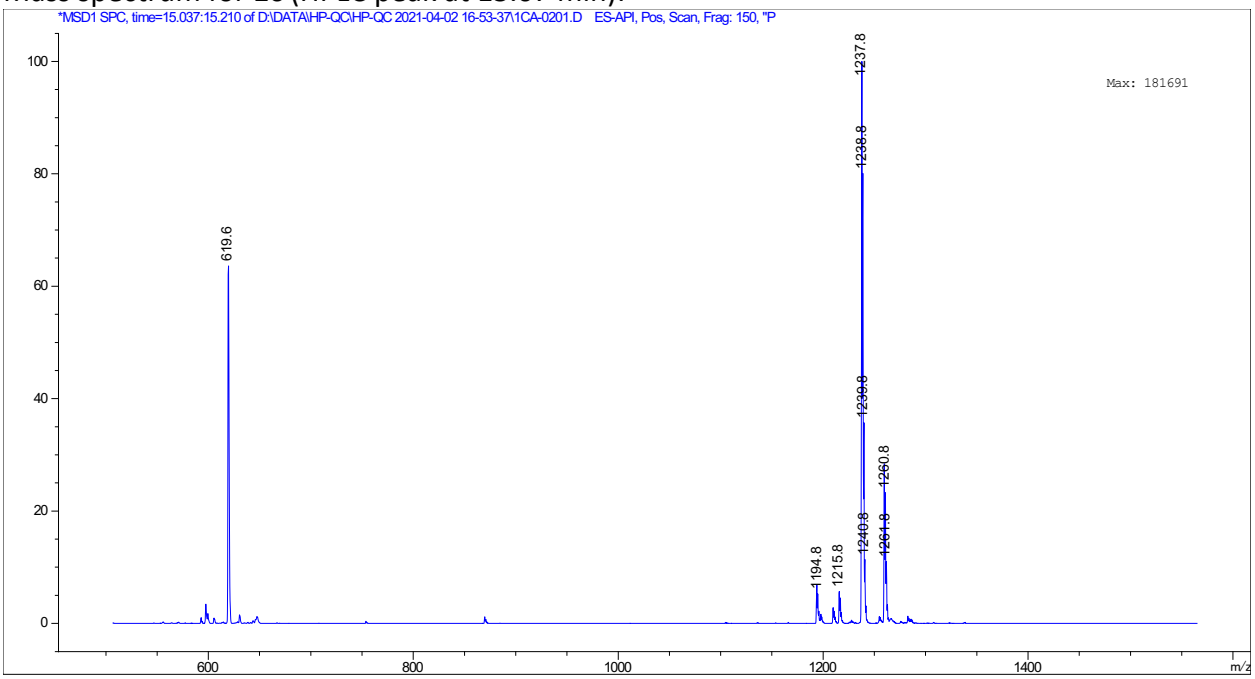
Mass spectrum for **1b** (HPLC peak at 15.01 min):



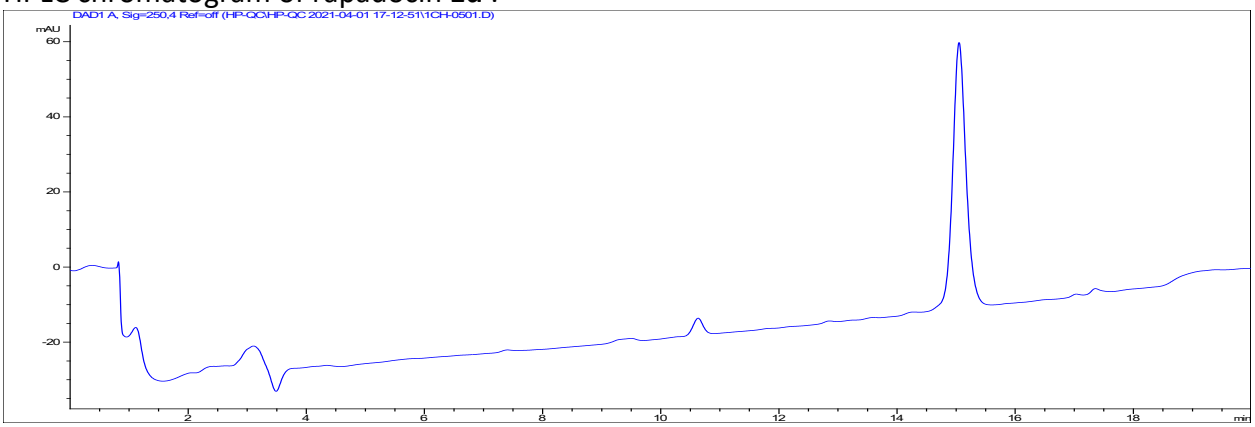
HPLC chromatogram of rapadocin **1c** :



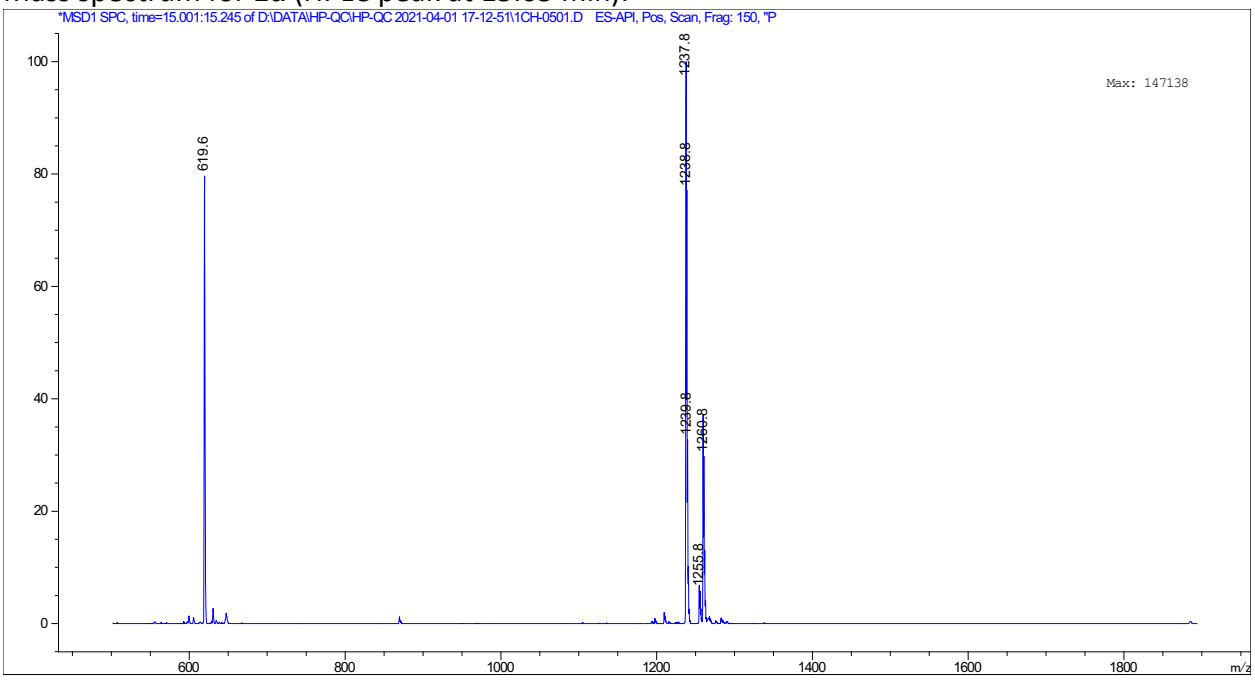
Mass spectrum for **1c** (HPLC peak at 15.07 min):



HPLC chromatogram of rapadocin **1d** :

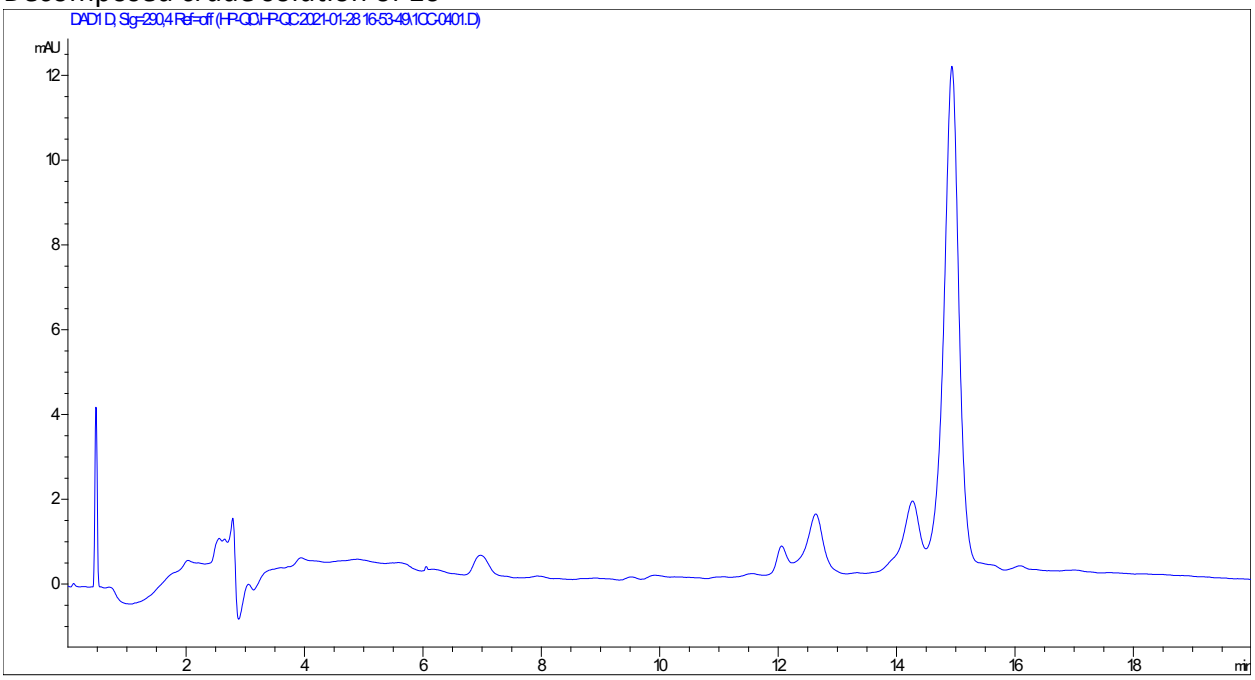


Mass spectrum for **1d** (HPLC peak at 15.05 min):

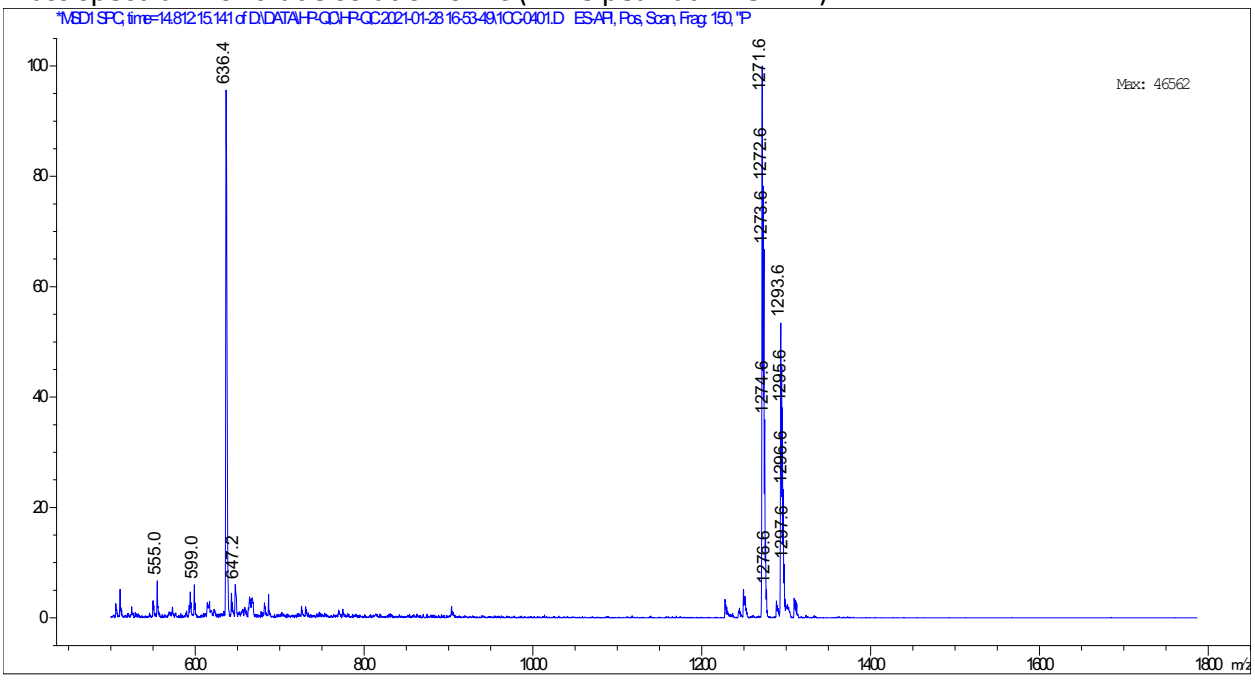


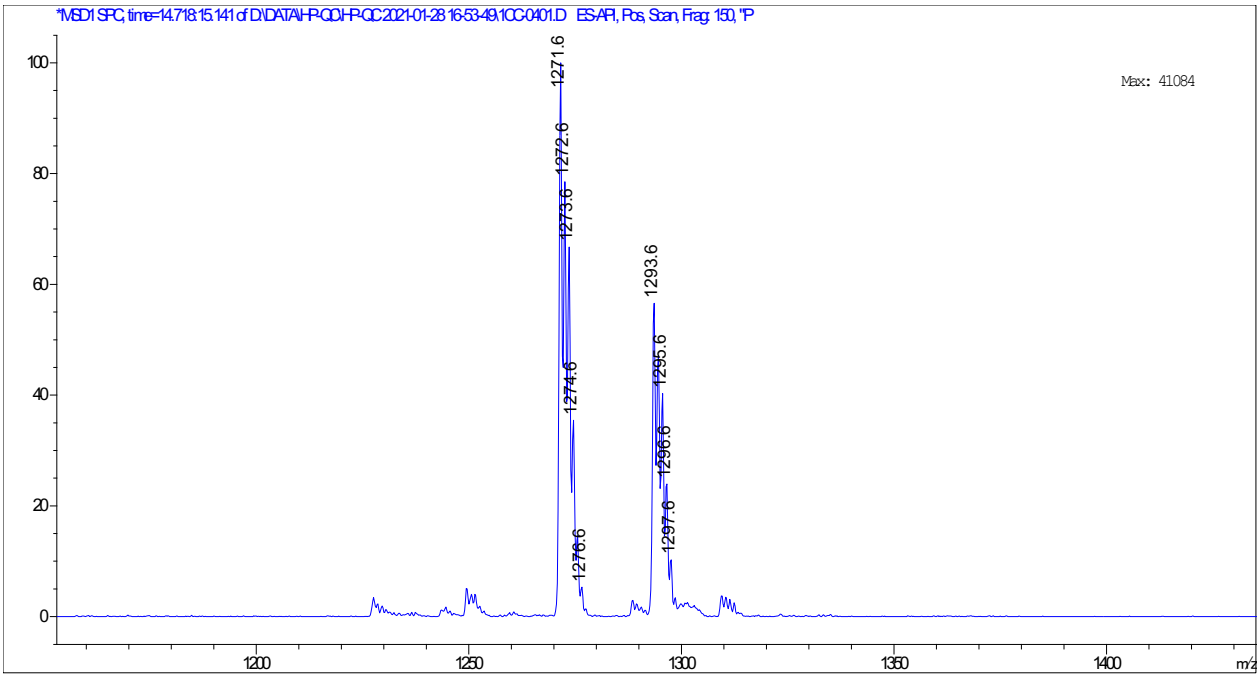
Chromatogram of HPLC of the 1c and 1d decomposed crude solution and MS of the HPLC peak

Decomposed crude solution of **1c**

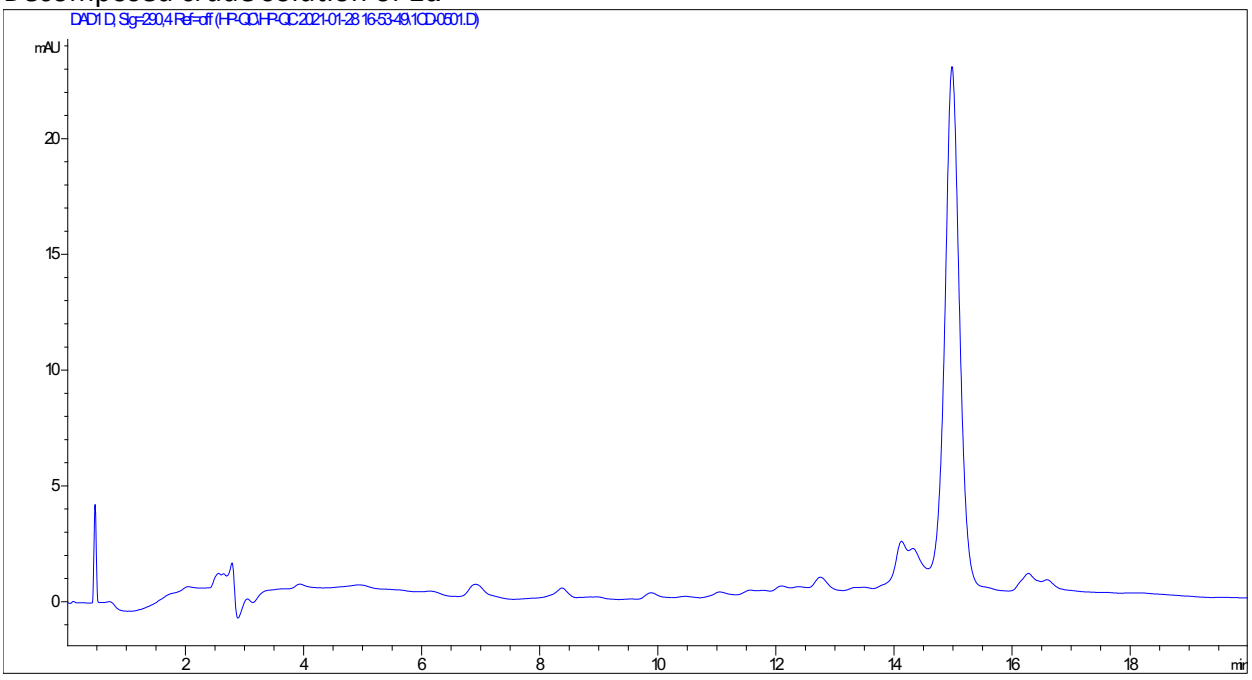


Mass spectrum for crude solution of **1c** (HPLC peak at 14.8 min):

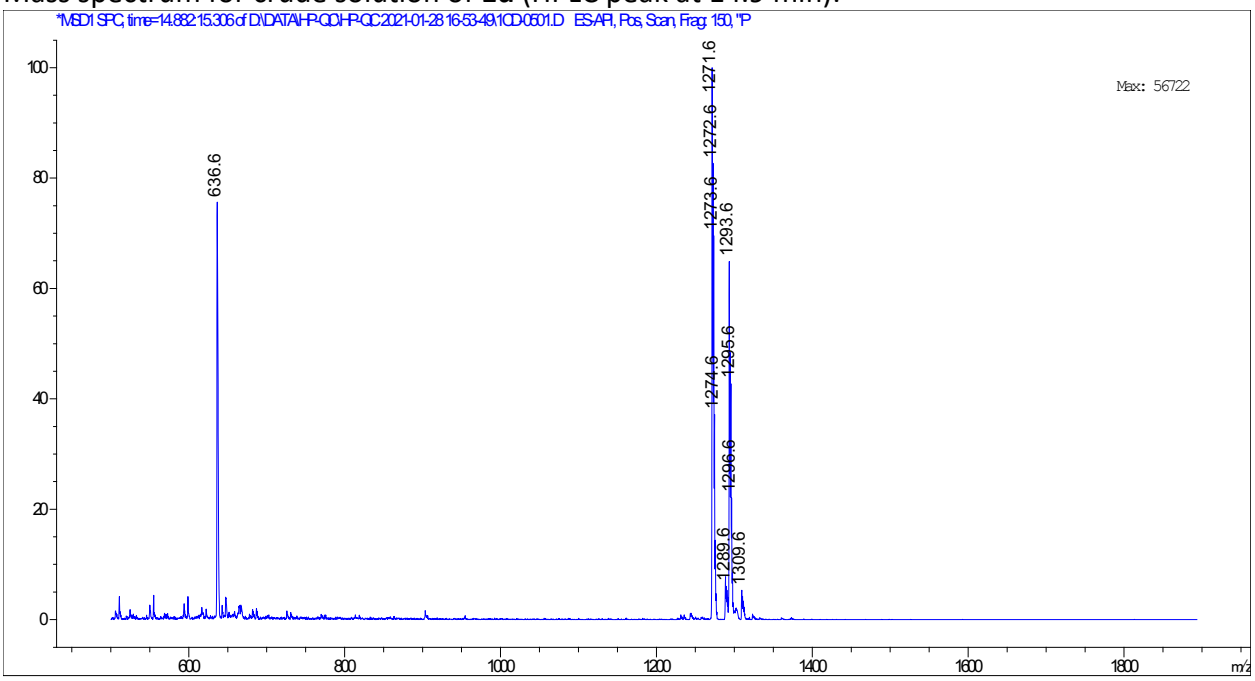


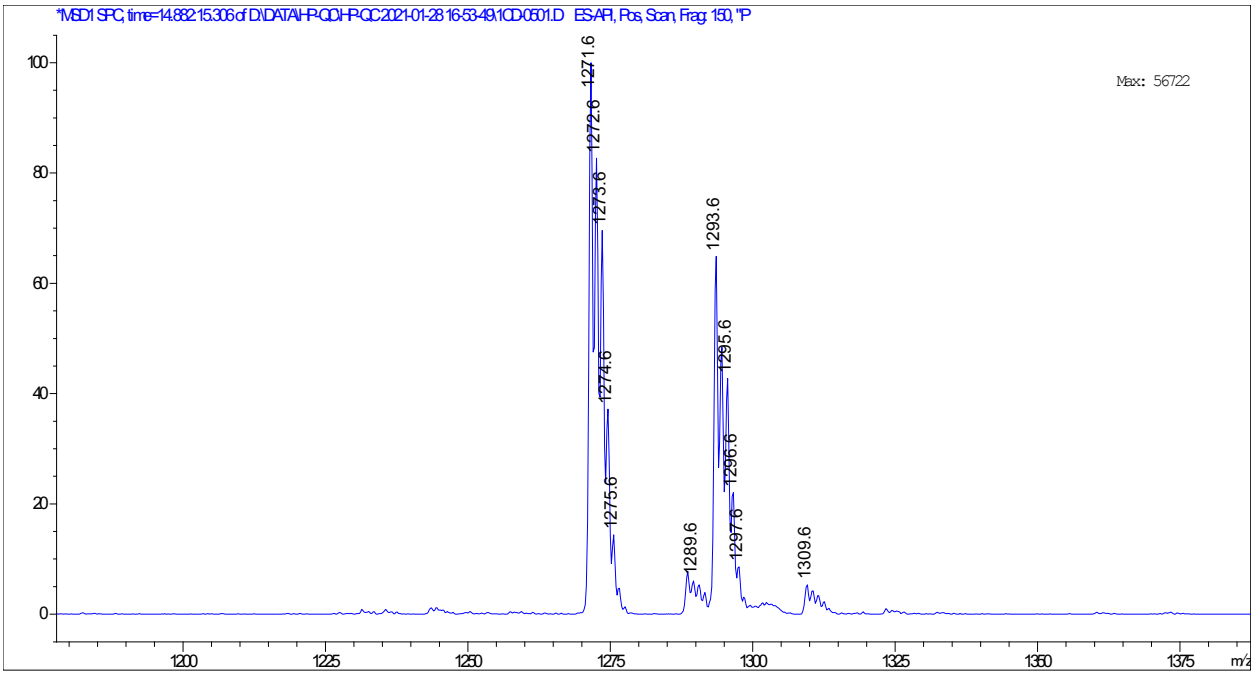


Decomposed crude solution of **1d**



Mass spectrum for crude solution of **1d** (HPLC peak at 14.9 min):





References

[1] K. Mahendrarajah, et al. *Anal. Biochem.* **2011**, *411*, 155-157.